THE GENETIC BASIS OF DIABETIC RETINOPATHY

by

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Diabetic retinopathy (DR) is a microvascular complication of diabetes and proliferative diabetic retinopathy (PDR) is the most vision threatening form of DR and the leading cause of blindness in individuals aged 20-74 years. Genetic endowment is thought to play a role in development of DR. The adenosine A2 receptor ($ADORA_{2A}$) is associated with decreased tissue impairment caused by inflammation, hypoxia, and oxidative stress, while vascular endothelial growth factor (VEGF) is associated with hypertension, vascular permeability, and neoangiogenesis, and is elevated in those with DR. The aim of this study was to determine if the $ADORA_{2A}$ or the VEGF genes are associated with prevalent baseline PDR (PBPDR) or prospective incident PDR (PIPDR) in participants of the Epidemiology of Diabetes Complications (EDC) prospective study of childhood onset type 1 diabetes. Two tagging single nucleotide polymorphisms (tSNPs) of the $ADORA_{2A}$ gene (rs2236624 and rs4822489) and 4 SNPs of the VEGF gene (rs2146323, rs833069, rs699947, rs10434) were genotyped and assessed for association with prevalence and incidence of PDR, while controlling for traditional covariates.

Univariate analysis of $ADORA_{2A}$ rs2236624 was associated with PBPDR [OR=1.68; (95%CI=1.11-2.54); p=0.03] and PIPDR [HR=0.17; (95%CI=0.04-0.69); p=0.01]. These associations were sustained in the multivariate analysis with rs2236624-CT being significantly associated with PBPDR [OR=2.17; (95%CI=1.24-3.81); p=0.03]. In the Cox analysis

rs2236624-CT [HR= 1.54; (95%CI=1.08-2.18); p=0.02] and rs2236624-TT [HR=0.10; (95%CI=0.01-0.72); p= 0.02] were significantly associated with PIPDR. Analysis of the rs4822489 tSNP found an association between the GT genotype and PIPDR in the univariate analysis [HR=1.53 ;(95%CI: =1.12-2.09); p=0.008], and in the multivariate analyses [HR=1.57 ;(95%CI: =1.13-2.17); p=0.0067] after controlling for covariates. To determine if there was increased risk associated with the 4822489 genotypes and PBPDR participants were stratified into late onset (PDR ≥25 years T1d duration), early onset (PDR<25 years T1D duration), and protected (No PDR during follow-up) groups. Those with the GT genotype had a significantly increased association [OR=1.93; (95%CI: =1.11-3.35); p=0.03] with early onset PDR compared to the protected group. The association between the GT genotype of the rs4822489 tSNP was only marginally significant [OR=1.73 ;(95%CI: =0.89-3.36); p=0.12] in the multivariate analyses after controlling for covariates. Furthermore univariately, the GG genotype of VEGF rs10434 was marginally [OR=1.3; (95%CI=0.75-2.28); p=0.14) associated with PBPDR but, multivariately significantly [OR=2.47; (95%CI=1.15-5.3); p=0054] associated with baseline PBPDR. Hypertension status [OR=3.93 (95%CI=1.98-7.8); p =<0.0001] low density lipoprotein [OR=1.01 ;(95%CI=1.004-1.02); p=0.0050] and duration [OR=1.21 (95%CI=1.12-1.3); p=<0.0001] were also significantly associated with PBPDR. None of the VEGF SNPs were associated with PIPDR. Additional analysis showed a significant (p=0.045) direct univariate association between the AG genotype of rs10434 and hypertension.

In the EDC population heterozygosity for the rs2236624 tSNP of the $ADORA_{2A}$ gene is associated with susceptibility to PDR while homozygosity for the (T) allele of rs2236624 tSNP is associated with protection form development of incident PDR. While homozygosity of the

VEGF rs10434 tSNP is associated with baseline prevalent PDR and heterozygosity for the rs10434 tSNP is associated with baseline prevalent hypertension.

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PREFACE

1.0   INTRODUCTION

1.1   DIABETES AND ITS COMPLICATIONS

Numerous advances have been made in understanding the etiology of human illness through mapping and analysis of the human genome. Researchers have discovered the genetic basis for various disease processes and have developed treatments based upon this information. Diabetes is a metabolic disorder, which results in hyperglycemia, and before the advent of medical therapy, death.   Due to advances in medical therapy, diabetic patients live longer; however, they often develop complications related to diabetes, such as, retinopathy, nephropathy, neuropathy, and cardiovascular disease.   These complications are the source of most of the illnesses and deaths of diabetic patients. As documented by the American Diabetes Association, greater than $170 billion of the healthcare dollars spent were attributed to medical and other costs related to diabetes in 2007.   Fifty-eight billion dollars of those expenses were used to treat diabetes complications and an additional $58 billion were used to cover costs related to absenteeism, work loss, unremitting disability and premature death.   Additionally, it has been estimated that the prevalence of diabetes will be more than twofold greater in 2050 than it was in 2005 (American Diabetes Association 2008).   Given the increase in the incidence and therefore the prevalence of diabetes, the cost of its care in the years to come will be much greater.

Determining the genetic basis of susceptibility to diabetes complications and/or genes associated with early onset of diabetes complications would offer nurses and other health care providers information that would facilitate not only early detection of at risk patients and implementation of appropriate therapies, but would also give healthcare providers the knowledge to educate their patients about their risks and modifications that may prevent or delay the onset of complications of their disease. After 15 to 30 years of type 1 diabetes (T1D) the majority of these patients will have some form of retinopathy (Stefansson, Bek et al. 2000; Sheetz and King 2002). Much of the literature has addressed genes associated with development or susceptibility to DR; however, few to date have addressed genes associated with why some patients differ in their length of DR free diabetes or why some patients with T1D progresses more rapidly to proliferative diabetic retinopathy, the more severe form of the vision corrupting disease.

1.2   DIABETIC RETINOPATHY

Diabetic retinopathy (DR) is a microvascular complication of diabetes characterized by micro-aneurysms, neovascularization with poorly formed weak vessels, vascular rupture, and bleeding in the retina. DR is the leading cause of new cases of blindness in individuals between the second and the seventh decades of life, and accounts for approximately 24,000 new cases of blindness annually (Centers for Disease Control and Prevention 2005; American Diabetes Association 2007). Approximately 20 million individuals in the United States have diabetes and most of these individuals will experience some form of DR during the course of their lives; however, those with type 1 diabetes (T1D) will experience a higher incidence of DR and will experience more vision threatening forms of DR with 90% developing DR once they have had

diabetes for 15 years and 60% developing proliferative DR once they have had diabetes for 20 years (Koulu, Movafagh et al. 2004).

1.3   PURPOSE

The purpose of this study was to investigate genes potentially associated with length of time from diagnosis of type 1 diabetes (T1D) to the development of proliferative diabetic retinopathy (PDR) as well as attainment of PDR.   This project focused on a population of patients diagnosed with T1D for a minimum of 25 years, who had been followed every 2 years for clinical assessment including development of complications, and investigated a possible genetic contribution to the development of PDR.

1.4   SPECIFIC AIMS

Specific Aim 1: Determine if a specific allele, specific genotype or haplotype generated for each candidate gene is associated with length of time to diagnosis PDR in individuals with T1D.

Specific Aim 2:   Determine if a specific allele, specific genotype and/or haplotype generated for each candidate gene is associated with attainment of PDR.

1.5   CONCEPTUALIZATION

The conceptual framework for this project is demonstrated in the figure below.

Genetic Endowment		Proliferative Diabetic Retinopathy
Genes involved with angiogenesis and vasculogenesis	**Covariates** • Age • Gender • BMI • Diabetes Duration • Glycemic control • Hypertension • Total Cholesterol • High Density Lipoprotein • Low Density Lipoprotein • Triglycerides • Ever Smoker	• Length of PDR free diabetes • Attainment of PDR
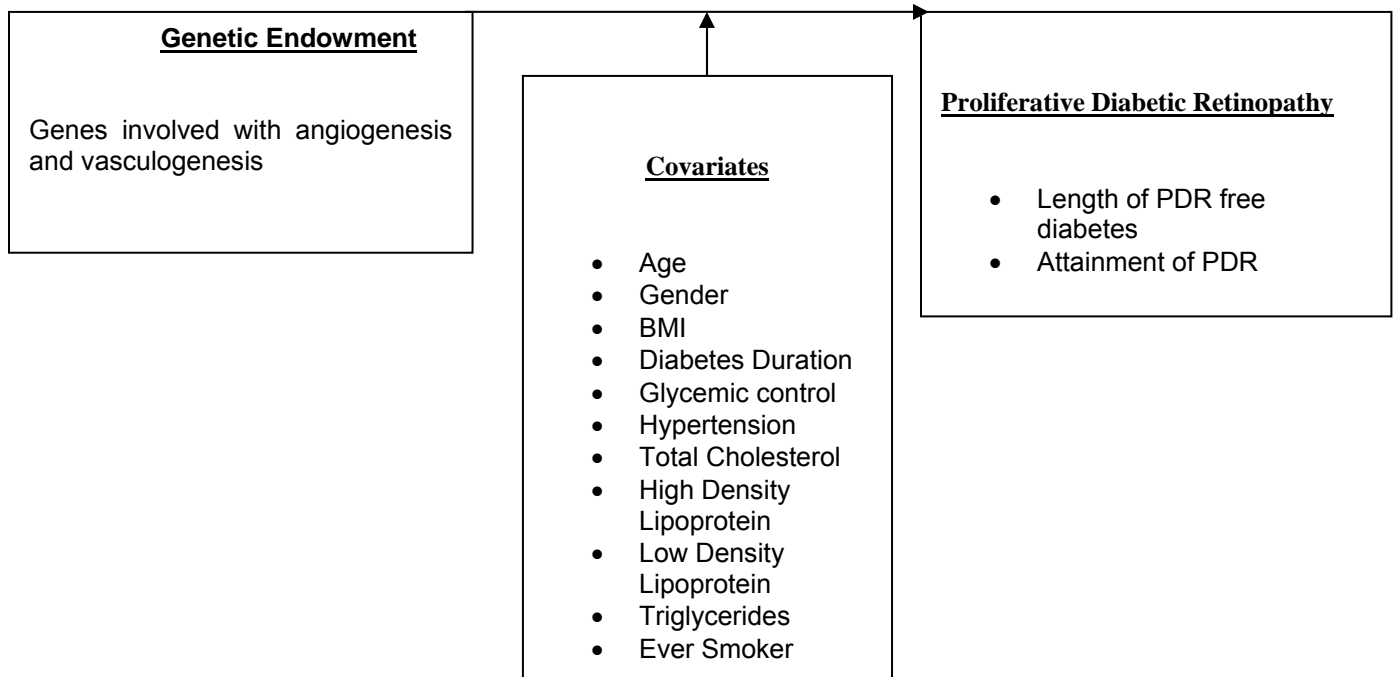
Figure 1: Conceptual Framework

2.0   BACKGROUND AND SIGNIFICANCE

This section includes information on diabetic retinopathy, information to support a genetic-based investigation into susceptibility to PDR, and information about angiogenesis and vasculogenesis and how it relates to PDR.

2.1   PREVALENCE AND PATHOPHYSIOLOGY OF DIABETIC RETINOPATHY

It is estimated that 2.4 million Americans have T1D (American Diabetes Association 2007) representing approximately one in every 400-600 children and adolescents in the United States or up to 10% of all confirmed cases of diabetes (Centers for Disease Control and Prevention 2005; American Diabetes Association 2007). Diabetic retinopathy is a microvascular complication of diabetes; therefore it makes sense to study genes involved with angiogenesis and vasculogenesis as plausible contributors to development of susceptibility to, or protection from the development of DR. Greater than 12,000, and possibly as high as 24,000 persons with diabetes, are diagnosed with retinopathy each year (American Diabetes Association 2007; National Eye Institute 2008). Diabetic retinopathy causes visual disturbances and is the most prevalent cause of new onset blindness in individuals 20-74 years of age (Centers for Disease Control and Prevention 2005; American Diabetes Association 2007). The pathogenesis of DR has four stages (National Eye Institute 2008). Prior to the first stage of DR and in association

with hyperglycemia, pericyte (contractile cell) cell death begins (Sheetz and King 2002). In stage one or Mild Nonproliferative Retinopathy the weakening of the vessels following the death of pericytes causes microaneurysms to develop in the microvasculature of the retina (National Eye Institute 2008). The second stage of DR or Moderate Nonproliferative Retinopathy is associated with impairment in the blood supply of vessels of the retina caused by clot formation (Petrovic, Hawlina et al. 2003; National Eye Institute 2008). The third stage of DR or Severe Nonproliferative Retinopathy is marked by increased impaired blood flow through the vessels supplying the retina and vascular neogenesis (formation of new vessels). The fourth and most severe stage of DR is Proliferative Retinopathy, which is marked by increasing vascular neogenesis near the vitreous humor. These new vessels are weak and malformed (National Eye Institute 2008). It has been estimated that after 15 years with diabetes 75-97.5% of individuals develop some form of DR (Klein, Klein et al. 1984; Sheetz and King 2002).   DR may worsen over time; however, not everyone progresses to proliferative DR. In the cases where it does progress these vascular changes may cause blindness by vitreous hemorrhage or by retinal detachment caused by fibrosis (Frank 2004; National Eye Institute 2008).

2.2   ANGIOGENESIS AND VASCULOGENESIS

A complex series of metabolic reactions utilizing different biologic pathways occur in the presence of hyperglycemia. Metabolic pathways that are activated during hyperglycemia influence the cellular environment and expose the cells to many toxic substances. Although this is a process that occurs in all individuals experiencing hyperglycemia, the non diabetic individual will respond by producing enough insulin to return blood sugar levels to a euglucemic state, thus

decreasing the non diabetic's exposure to hyperglycemia and the physiologic responses that include the production of substances toxic to the cells. In contrast to the response of the individual without diabetes, individuals with diabetes have blood sugar levels that are elevated more frequently and for more extended periods of time. Some claim that these glycemic elevations are responsible for changes in the extracellular matrix resulting in capillary basement membrane thickening (Akbari and LoGerfo 1999). Furthermore, when the cells are exposed to hypoxia or toxic substances the cells may become injured, dysfunctional and die. The toxic substances that the cells are exposed to include sorbitol (increasing osmotic pressure), hydrogen peroxide, lactate, toxic levels of glucose, reactive oxygen species, and advanced glycation end products. This exposure may lead to an increase in inflammation. Furthermore hypoxia and vascular cell injury may result in pericyte cell death, and with progression, formation and rupture of aneurisms (Chowdhury, Kumar et al. 1992; Sheetz and King 2002; Boehm, Lang et al. 2003; Kumaramanickavel, Sripriya et al. 2003; Whikehart 2003). Capillary rupture results in plasma lipids and proteins being deposited over time resulting in the formation of solid exudates (Sheetz and King 2002). The rupture of these vessels triggers increased hypoxia and vascular impairment stimulating a pathophysiologic response which attempts to repair the injured vascular system of the eye. Unfortunately the new vessels are weak and ill formed. As mentioned above, in individuals without diabetes this feedback mechanism is not activated as frequently or for extended periods of time, therefore this pathologic growth of new vessels is not activated in the individual without diabetes (Boehm, Lang et al. 2003).

The abnormal vascular growth and the physiologic and pathophysiologic mechanisms mentioned above have lead to the postulation that one's genetic endowment from a myriad of genes, each having multiple possible variants with their own pleiotropic effect may influence

one's susceptibility to complications of diabetes, particularly retinopathy, stage of DR attained and length of PDR free diabetes. Because the primary debilitating clinical manifestation of diabetic retinopathy is abnormal vascular growth it makes sense to explore the relationship between multiple genes in the vasculogenic and angiogenic pathway for an association between their variants and the development of proliferative diabetic retinopathy. It is also plausible that various combinations of these variants create a biologic environment that produces individual susceptibility to or protection from the development of diabetic retinopathy, non-proliferative diabetic retinopathy and proliferative retinopathy, as well as influencing the length of PDR free diabetes.

The mechanisms involved with vasculogenesis and angiogenesis are not completely understood. At one time angiogenesis was identified as being the development of newly formed blood vessels arising from previously formed blood vessels, while vasculogenesis was identified as being the formation of blood vessels from single predecessor cells called angioblasts during embryogenesis. Investigators now believe that the lines between these two processes are not so clearly drawn (Auerbach and Auerbach 2001).

The process of angiogenesis and vasculogenesis is required for the development and sustenance of healthy and pathologic tissues (Hackett, Ozaki et al. 2000). The vasculogenic and angiogenic processes involved in the development of the vascular system within the retina have been described (Hughes, Yang et al. 2000). The vasculature of the retina is composed of a very thin, small complex of capillaries sandwiched between an exterior and interior layer of vessels. When the retina is in the presence of uncontrolled hyperglycemia, toxic substances, such as sorbitol and advanced glycation end products (AGEs), are produced. Investigators postulate that these substances cause pericyte cell death, resulting in the development of microaneurysm

formation. Ex-vivo experiments have illustrated that pericyte cell death is followed by the rapid growth of endothelial cells, a process which has lead investigators to postulate that the death of pericytes leads to the initiation of proliferative and nonproliferative diabetic retinopathy (Hammes, Lin et al. 2002; Wauiter and Schmidt 2004). When aneurysms form and rupture the areas that the ruptured vessel/s feed are deprived of oxygen, resulting in a hypoxic state. Hackett et al have demonstrated that neovascularization develops in response to the ischemic retina (Campochiaro 2000; Hackett, Ozaki et al. 2000). While the exact mechanisms involved in the activation of neoangiogenesis are not clearly understood, it is associated with the expression of cytokines, extracellular matrix proteins, vasoactive peptide, integrin receptors, and angiopoeitins (Knott, Robertson et al. 1999; Jones 2001; Pepper, Mandriota et al. 2001; Rodriguez and Brooks 2001; Walsh and Fan 2001).

2.3   RATIONALE FOR INVESTIGATING THE GENETIC BASIS OF
DIABETIC RETINOPATHY

Animal and human models provide empirical evidence that supports the possibility of a genetic susceptibility in some individuals toward developing DR. This complication of diabetes tends to cluster in racial groups and families of individuals with known diabetes. Family Evidence: Using a United States population with diabetes drawn from the third National Health and Nutrition Examination Survey investigators found that among individuals with type 2 diabetes, Mexican Americans were more likely to develop all stages of DR than were Non-Hispanic Blacks, and Non-Hispanic Blacks were more likely to develop all stages of DR than were Non-Hispanic Whites, while Non-Hispanic Blacks without diabetes were less likely to

develop any form of retinopathy than Mexican Americans or Non-Hispanic Whites (Harris, Klein et al. 1998). Other investigators have reported conflicting results with regard to the prevalence of DR among Hispanic verses Non-Hispanic Whites and suggest that the prevalence between the two groups is actually similar (Tudor, Hamman et al. 1998). A more recent study using data from the 2001 Behavioral Risk Factor Surveillance System (BRFSS) found that Whites had a lower prevalence of DR than Blacks, Hispanics, or Native Americans, while Asians had a higher prevalence of DR than all four previously mentioned groups and Pacific Islanders had the highest prevalence of DR among all of the groups (McNeely and Boyko 2005). Other investigators have estimated that among individuals with T1D prevalence of any DR is 75% as opposed to 82% in Black and Whites respectively. While prevalence of PDR is 30% as opposed to 32% in Black and White individuals with T1D, respectively (Roy, Klein et al. 2004). The NIH and estimates that among all individuals with known diabetes 59.2% of Hispanics, 36.08 % of Whites, 31.19% of Blacks and 44.25% of all others have prevalent DR (National Eye Institute 2002). Family Evidence: The Diabetes Control and Complications Trial (DCCT) found that some patients with poor glycemic control never developed DR, while other patients who maintained good glycemic control did develop DR. This discordance between the expected physiologic response based on glycemic control and the actual development of DR in some individuals suggests that there must be some other basis for this phenomenon other than glycemic control and length of time with diabetes (Diabetes Control & Complications Trial 1993). Subsequently, a family clustering study of DR was conducted using the same population. This study noted a positive relationship between DR severity and family clustering. In other words severity of DR appeared to have a similar pattern within the same family clusters (Diabetes Control and Complications Trial 1997). This phenomena, supports the probability of a genetic based etiology for developing DR.

Additionally a family clustering study of South Indian families with at least 2 siblings with diabetes was conducted between 1991 and 1997. A total of 355 siblings participated in the study. DR was diagnosed in 21.4% of these siblings and there was a 35.3% concordance in their diabetic siblings while in the 78.6% of siblings without DR only11.2% in their diabetic siblings had DR (Rema, Saravanan et al. 2002)

In a Mexican American population, after controlling for duration of diabetes and glycemic control, investigators found that more vision threatening forms of DR tended to cluster in families after they combined individuals with moderate non-proliferative DR and individuals with PDR into one group (Hallman, Boerwinlle et al. 2007). These finding were supported by a study of 767 families (n=2368) with diabetes. These investigators found that severity of DR tended to aggregate within families (Arar, Freedman et al. 2008). In all these findings support a rationale for exploring a genetic basis for DR or severity of DR attained.

Genes within the angiogenesis and vasculogenesis pathways mentioned above will be investigated for an association between the development of diabetic retinopathy and variants of identified genes. Variants to be investigated in this project will be tagging SNPs with a minor allele frequency of 20% or greater and are illustrated in the table noted below:

Table 1: Variants for this project based on their involvement in angiogenesis and vasculogenesis.

GENE	FUNCTION	HAPMAP TAGGED SNPS	REFERENCES
Vascular Endothelial Growth Factor (VEGF)	Associated with vascular permeability, controls/induces neoangiogenesis and is elevated in DR. Upregulation induced by advanced glycation end products.	**(2)** rs10434 rs833069 rs2146323	(Duh and Aiello 1999) (Caldwell, Bartoli et al. 2003) (Nakagawa, Chen et al. 2000) (Lu, Kuroki et al. 1998) (Perrin, Konopatskaya et al. 2005) (HapMap 2006)
Adenosine A2 Receptor (ADORA$_{2A}$)	Modulates activity of vascular cells including protection from stress such as ischemia and limits the inflammatory response and cell injury in animal models.	**(2)** rs4822489 rs2236624	(HapMap 2006) (McKusick 2006)

2.4   VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

CHROMOSOME 6P12

In 1948 Michaelson postulated that there was an unknown factor that accounted for the growth of new vessels in the retina (Michaelson 1948); however, vascular endothelial growth factor was not isolated and identified as being a necessary component of normal and pathologic angiogenesis (vascular growth) via initiation of vascular endothelial cell proliferation, migration, and tube formation until 1991 (Favard, Moukadiri et al. 1991; McKusick 2006; Penn, Madan et al. 2008).   Retinal hypoxia, is a component of the pathological response to the hyperglycemic state and VEGF is up-regulated in the presence of hypoxia (Campochiaro 2000; Hackett, Ozaki et al. 2000; Perrin, Konopatskaya et al. 2005).   Oxidative stress and inflammation are also part of the pathologic response to hyperglycemia and these processes also result in the up-regulation of VEGF (Favard, Moukadiri et al. 1991), which is a primary trigger for the neoangiogenesis and the weak leaky vessels characterized by DR (Perrin, Konopatskaya et al. 2005).   Furthermore, VEGF has been well defined in both animal and human models as contributing to the development of DR. Starting n the 1990s animal and human studies identified an association between VEGF and the ocular fluid of rats, primates and humans with DR (Aiello, Avery et al. 1994; Miller, Adamis et al. 1994; Nakagawa, Chen et al. 2000). Subsequently genetic association studies were conducted in Japanese, East Indian and Caucasian populations, all of which reported significant associations between VEGF variants and the prevalence of some form of DR. These associations have been confirmed in individuals with T1D and T2D.   In 2002 investigators

reported an association between DR and the 5'-untranslated region (C-634) of the VEGF gene in a population of 268 Japanese patients with T2D (Awata, Inoue et al. 2002). Later, investigators in England reported an association between the VEGF-460C polymorphism and development of PDR in a Caucasian population of individuals with T1D and T2D (Ray, Mishra et al. 2004). In a population of 210 T2D patients of east Indian ancestry investigators found that the C(-7)T, the T(-1498)C, and the C(-634)G, polymorphisms of the VEGF gene were significantly associated with DR (Suganthalakshmi, Anand et al. 2006). Interestingly, Nakamura and colleagues found an association between the -2578AApolymorphism but not the C(-634)G, polymorphism of the VEGF gene and prevalence of DR (Nakamura, Iwasaki et al. 2008). Investigators of the DCCT provided evidence that three SNPs (rs3025007, rs3025020 and rs3025035) of the VEGF gene were associated with severe retinopathy in a univariate model; while eight SNPs (rs699947, rs833070, rs2146323, rs3035007, rs3025010, rs3025020, rs3025021, rs3025028) were associated with severe retinopathy in the multivariate analysis (Al-Kateb, Mirea et al. 2007). Finally, investigators in England provided evidence that two SNPs in the promoter region (rs13207351-160 CC genotype and the -152 AA genotype, and rs1570360-116 AA genotype) were significantly associated with PDR in a mixed T1D and T2D population (Churchill, Carter et al. 2008).

2.5   ADENOSINE 2A RECEPTOR (ADORA$_{2A}$)

It is postulated that oxidative stress (OS) caused by long term exposure to hyperglycemia may be responsible for some of the tissue damage associated with complications of diabetes. OS is elevated in the retina of individuals with diabetes and may contribute to the

development of DR (Pan, Zhang et al. 2008). In the presence of OS hydrogen peroxide is formed in the cytosol rendering the vascular endothelial cells more permeable (Hecquet, Ahmmed et al. 2008). In contrast adenosine, derived from ATP metabolism, is a powerful physiologic mediator, thought to modulate cellular damage and the resulting tissue injury caused by biologic stressors. This modulation by adenosine is directed by the adenosine receptors (AR), with the Adenosine A1 receptor having a proinflammatory response to tissue injury while the adenosine A2 receptor (ADORA2) restricts inflammation and guards tissues from further damage (Salmon, Brogle et al. 1993; McKusick 2006). Investigators in Italy provided evidence that ARs have variability in function and the subsequent responses to ischemia found in the rat brain (Trincavelli, Melani et al. 2008). Other animal studies have provided evidence that $ADORA_{2A}$ protects the heart from ischemic reperfusion injury by reducing the generation of ROS, by limiting mitochondrial damage and by guarding against apoptosis (Xu, Mueller et al. 2005; Xu, Park et al. 2005). $ARORA_{2A}$ also contributes to activation of physiologic pathways that protect kidney cells from injury due to ischemia and reperfusion (Yonehana and Gemba 1999). Many of $ADORA_{2A}$'s affects in humans have not yet been elucidated. The role $ADORA_{2A}$ plays in ameliorating the effects of biologic stressors in animals coupled with the fact that adenosine's effects are modulated by the ARs makes $ADORA_{2A}$ gene a plausible candidate gene for exploring susceptibility or protection from the development of DR. It may also affect the severity of DR attained. The $ADORA_{2A}$ receptor gene is located on chromosome 22q11.2. This gene has been further characterized in the manuscript "$ADORA_{2A}$ is Associated with Proliferative Diabetic Retinopathy in Type 1 Diabetes".

3.0   PRELIMINARY STUDIES

Preliminary analysis of each SNP was conducted upon receipt of the assay from ABI to assess quality of the assay. Additionally after each SNP was genotyped additional analysis were conducted to assess whether or not the SNP genotypes were in Hardy-Weinberg equilibrium. Please see Table 2 for the $ADORA_{2A}$ tSNPS and Table 3 for the VEGF SNPs.

Table 2: $ADORA_{2A}$ Allele Frequencies and Genotype Frequencies

$ADORA_{2A}$			
Rs2236624		Rs4822489	
Allele Frequencies		Allele Frequencies	
C	0.79	G	0.61
T	0.21	T	0.39
Genotype Frequencies		Genotype Frequencies	
CC	308	GG	195
CT	154	GT	212
TT	24	TT	84

Table 3: VEGF Allele Frequencies and Genotype Frequencies

VEGF			
RS699947		RS833069	
Allele Frequencies		Allele Frequencies	
A	0.45	C	0.35
C	0.55	T	0.65
Genotype Frequencies		Genotype Frequencies	
AA	101	CC	66
AC	233	CT	212
AA	148	TT	212
RS2146323		RS10434	
Allele Frequencies		Allele Frequencies	
A	0.32	A	0.46
C	0.68	G	0.54
Genotype Frequencies		Genotype Frequencies	
AA	52	AA	102
AC	210	AG	241
CC	227	GG	141

4.0   RESEARCH DESIGN AND METHODS

Additional Research Design and Methods details can be found within the manuscripts contained within this document.

4.1   RESEARCH DESIGN

The design of specific aim 1 uses a nested association approach focusing on the genes which may be associated with the time of PDR free diabetes. Survival analysis methods will be used to assess the participants in biennial increments from time of diagnosis of diabetes until onset of PDR.

The design of specific aim 2 uses a case control association study approach focusing on the genetic susceptibility to or protection from proliferative diabetic retinopathy. The cases will be comprised of individuals who have been affected with Type 1 diabetes (T1D) for a minimum of 25 years who have documented proliferative diabetic retinopathy. The controls will be comprised of individuals who have been affected with T1D for a minimum of 25 years who have NOT developed proliferative diabetic retinopathy. Please see Table 4 for counts and frequencies related to DR status of EDC study participants.

Table 4: Development of diabetic retinopathy in the EDC study

IDDM Duration at time	Number with DR	Percent of participants
5-9	44	2%
10-14	104	7%
15-19	147	22%
20-24	113	38%
25-29	102	77%
30+	68	68%
30+	79***Never developed	

4.2   SAMPLE

The population for this study consists of 496 (75.4%) of the 658 participants of the Pittsburgh Epidemiology of Diabetes Complications (PEDC) Study for whom banked DNA was available. These participants offer a unique opportunity to investigate the genetic contribution to the development of PDR, because there is extensive clinical data available for them as well as banked DNA. The clinical data were derived from clinical assessments which were conducted at baseline then approximately every 2 years over a 20-year period. The assessments included, presence of complications, including a retinal exam during which fundus photos were taken; blood pressure; duration of diabetes complications; body mass index; glycosylated hemoglobin; total cholesterol; low density lipoprotein; high density lipoprotein; triglycerides; ever smoker status; HbA1C; homocysteine; antibodies to oxidized LDL; water and lipid soluble antioxidants including uric acid; α and γ tocopherol; thiols and cystatin-C; apoB, cellular adhesion molecules such as ICAM-1 and VCAM-1; P and E selectin; IL-1, IL-6; IL-8m, TNF α ; TGFβ; CTGF; complete lipid profile; PAI-1; fibrinogen; adiponectin; a CBC; and C reactive protein. Females represent 49.5% of the study population and the racial representation is similar to that of the Allegheny County T1D registry which consists of 4.6% African American, 95.4% Caucasian

and no representation from other ethnicities, while the EDC participants consists of 2% African-American and 98% Caucasian.

Table 5: Retinal assessment categories

Category 1	Category 2	Category 3	Category 4
No Retinopathy	Early Background Retinopathy	Advanced Retinopathy	Proliferative Retinopathy
Grade 10 in Both Eyes	Grade 20 or 30 in Either Eye	Grade 40 or 50 in Either Eye	Grade 60 or > in Either Eye

4.3   RECRUITMENT

The recruitment pool consisted of members of the Children's Hospital of Pittsburgh diabetes registry which treated 70% of all T1D patients in Allegheny County. Potential participants received a letter inviting them to have a physical examination and to complete several questionnaires. There were 979 eligible participants, 658 (67.2%) of whom participated in the entire EDC evaluation process (Kostraba, Klein et al. 1991; Costacou, Chang et al. 2006). The current study, "Genetic Basis of Diabetic Retinopathy" (GBDR) consists of 496 (75.4%) EDC participants.   The remaining 24.6% of the participants were excluded due to lack of genetic material.

Inclusion criteria for the PEDC and GBDR studies:

- Childhood-onset of type 1 diabetes

- Live within 100 miles or 2 ½ hour drive to Children's Hospital of Pittsburgh

- Banked DNA

Exclusion criteria for the EDC and GBDR studies:

- Adult onset diabetes mellitus of any type

- Lack of availability of banked DNA due to missed visit

This study was approved by the Institutional Review Board at the University of Pittsburgh and written informed consent was obtained from participants prior to baseline data collection.

Measures/Instruments: DNA samples: DNA was extracted from leukocytes and banked using a unique numerical identification system. These DNA samples are stored in 1X TE buffer, and are of adequate concentration to allow for the collection of the genotypes required for this study and were made available for the purpose of this study through Dr. Trevor J. Orchard and Dr. Robert E. Ferrell.

4.4   RESEARCH SETTING

Genetic Laboratory: All genotyping for the GBDR study was conducted in Dr. Yvette P. Conley's Laboratory located at 3500 Victoria Street on the fourth floor of the University of Pittsburgh school of Nursing. It is comprised of 4 areas:

1. A pre-PCR room

2. A post-PCR room

3. A culture room

4. A cold storage room

5. A room temperature storage room

These rooms are housed in a 3200 square foot area. The lab provides an ABI 377 automated sequencer/genotyper with accessory computer equipment and software for data analysis, the WAVE® Nucleic Acid Fragment Analysis System from Transgenomic which carries out dHPLC, a Turner Designs Luminometer, myriad horizontal and vertical electrophoresis units and power packs, a computerized camera and gel documentation software, centrifuges, a spectrophotometer, -80 degree freezers, five 96 well thermocyclers and an ABI 7000 TaqMan® real time PCR and allele discrimination assays.

4.5   PROCEDURES

Genotype Data Collection Methods:

Bioinformatics for SNP selection: Tagging single nucleotide polymorphisms (tSNPs) for each gene were selected using the HapMap database (NCBI build 35) (hapmap.org) (HapMap, 2008) using criteria of a minor allele frequency of 20% or greater and an r^2 cut off of .80. An ABI Prism® 7000 Sequence Detection System was used to conduct allelic discrimination using TaqMan® assays. This is a highly automated, high throughput genotyping method for SNPs. Primers flank each polymorphism and PCR is conducted using subject DNA. The main difference between TaqMan® PCR and general PCR is the use of a probe that is labeled with a reporter and a quencher dye that recognizes a specific allele of a SNP. Each allele of a SNP has its own probe with its own reporter dye. If the subject's DNA is homozygous for one allele of the SNP, only the probe for that allele will hybridize and only the reporter dye for that allele will be liberated and measured by the ABI Prism® 7000 Sequence Detection System. A heterozygote will have both probes hybridize and both reporter dyes measured. Using this method, each SNP can be genotyped accurately and quickly.   Assay-on-demand kits or made to order kits were purchased from ABI for each of the selected SNPs.   These kits supplied the primers, probes, and reagents for the genotyping of each of these SNPs. In addition the PCR conditions for each SNP had been established by ABI and were supplied with the purchase of the assays. The Taqman Genotyping conditions are noted below. When the results yielded poor separation using the normal protocol, the extended protocol was used and generally resulted in improved separation and allelic discrimination.

TaqMan Conditions- Normal Protocol:

- Step 1: 50ºC for 2 minutes UNG enzyme activation

- Step 2: 95ºC for 10 minutes AmpliTaq Gold

- Step 3: 95ºC for 15 seconds to denature DNA

- Step 4: 60ºC for 1 minute for annealing and strand extension

- Step 5: Repeat from Step three 30 times

TaqMan Conditions- Extended Protocol:

- Step1: 50ºC for 2 minutes UNG enzyme activation

- Step 2: 95ºC for 10 minutes AmpliTaq Gold

- Step:3 95ºC for 15 seconds to denature DNA

- Step 4: 58ºC for 1 minute and 30 seconds for annealing and strand extension

- Step 5: Repeat from step three 50 times

- Table 6: TaqMan Genotyping Conditions and Protocol for ADORA$_{2A}$:

Table 6: TaqMan Genotyping Conditions and Protocol for ADORA$_{2A}$

Gene ADORA2A	Assay on Demand (20X) Concentration	Made To Order (40X) Concentration	Genomiphied DNA and H$_2$O 5:100(ul)	Universal PCR Mix (ul)	Assay (ul)	TaqMan Conditions
Rs2236624		Yes	5.7ul	6.25	0.3125	Extended
Rs4822489	Yes		5.7ul	6.25	0.625	Extended

Table 7: TaqMan Genotyping Conditions and Protocol for VEGF

Gene VEGF	Assay on Demand (20X) Concentration	Made To Order (40X) Concentration	Genomiphied DNA and H_2O 5:100(ul)	Universal PCR Mix (ul)	Assay (ul)	TaqMan Conditions
Rs699947		Yes	5.7	6.25	0.3125	Normal
Rs2146323		Yes	5.7	6.25	0.3125	Normal
Rs8333069		Yes	5.7	6.25	0.3125	Normal
Rs10434		Yes	5.7	6.25	0.2125	Extended

4.6   DATA MANAGEMENT

SNP genotypes were placed in Excel spreadsheets using the unique identifiers assigned to the specimen prior to receipt of the specimen. The genotype data and the existing EDC data were merged by a unique case identification number using SAS 9.1.3 statistical software (SAS Institute Inc 2000-2004). The merged data was maintained in a separate database on a password protected computer.

4.7   DATA ANALYSIS

Statistical analyses were also conducted using SAS version 9.1.3 statistical software. Prior to the conduct of primary analyses to address the specified research aims, a detailed preliminary analysis was conducted, and included a descriptive analysis of collected data, a summarization of data and the use of inferential and graphic exploratory data analytic techniques to identify any data abnormalities. Categorical variables, such as PDR status, gender, race,

hypertension status, ever smoker status, and genetic endowment with genotypes of a particular tagging SNP, were described using frequency tables (with frequency counts and percentages) and contingency. Continuous variables such as duration of diabetes and age, were assessed using frequency distributions, histograms, box plots, normal probability plots, and/or stem and leaf plots. The shape of the distribution of continuous variables was described in terms of degree of normality, skewness kurtosis. The mean and standard deviation of continuous variables were used to describe the variable's central tendency (i.e. location) and dispersion. The presence of univariate and multivariate outliers was assessed using Box plots, histograms, and normal probability, detrended normal probability, and bivariate scatter plots. Multicollinearity was assessed using variance inflation factor, tolerance indices, and collinearity diagnostics (i.e., condition indices and variance decomposition proportions). Variance inflation factor values greater than 10, tolerance values less than 0.10 and a conditioning indices more than 30 for a given predictor variable paired with at least 2 variance decomposition proportions greater than .50 were deemed to be indicative of multicollinearity. and/or collinearity values greater than or equal to 25 were judged to be indicative of multicolinearity. Data transformations were applied as necessary (i.e., squared, quadratic and cubed).

Survival analysis and ordinal logistic regression methods were used in the primary analysis of the data. Prior to the use of these methods, key assumptions specific to the method were evaluated (e.g., linearity of the logit, proportional odds assumptions, proportional hazards assumption). Survival analysis methods were used to assess Specific Aim 1. Initially a crude analysis of each SNP was investigated for its association with length of time to diagnosis of PDR using Kaplan-Meier curves. Univariate Cox proportional hazards regression was used to assess the predictive association between the SNP genotypes and incident PDR. When controlling for

covariates, multivariate Cox proportional hazards regression methods were employed. The covariates were identified in the preliminary analysis using stepwise Cox proportional hazards regression methods. Covariates found to be significant (in the preliminary analysis were included in the multivariate analysis along with SNP's genotypes to investigate their joint association with length of time to development of PDR.

For Specific Aim 2 proportional odds logistic regression analysis was used to investigate the association between prevalent PDR attainment and the SNPs listed Table 1. Subjects were categorized by PDR status. Initially, each SNP was be analyzed for its crude (unadjusted) association with PDR attainment using exact chi square analysis to assess independence and to accommodate possible sparseness of cells. The genotypes of the SNPs were assessed using backward selection bivariate logistic regression and a .10 level of significance for covariate removal. Covariates were selected using univariate logistic regression. Lastly, the SNPs were be analyzed for PDR attainment taking the covariates into consideration using stepwise logistic regression analysis and a significance level of .05 for covariate entry and .10 for variable removal. Model assessment (i.e., residual analysis, identification of influential observations0 was performed for all proportional odds and proportional hazards models estimated..

5.0   PUBLICATIONS

This section consists of two abstracts submitted for publication. The first abstract reports the findings of the ADORA$_{2A}$ gene and gene and its association with PDR in the EDC derived GBDR population, while the second abstract reports the results of the investigation of the VEGF gene and its association with PDR in the EDC derived GBDR population.

5.1   ABSTRACT #1: ADORA$_{2A}$

Purpose: The ADORA$_{2A}$ receptor ameliorates many of the deleterious physiologic effects associated with tissue injury and biologic stressors. It may also ameliorate some of the biologic stressors experienced by individuals with diabetes.   Thus, we investigated cross-sectionally and prospectively association between the ADORA$_{2A}$ gene and proliferative diabetic retinopathy (PDR) in a sample of patients with type 1 diabetes (T1D).

Methods: Study participants were from the Pittsburgh Epidemiology of Diabetes Complications (EDC) prospective study of childhood onset (<17 years) T1D, (mean age 28 years, mean diabetes duration 19 years at enrollment).   Participants underwent stereoscopic images of the retinal fundus at baseline (1986-1988) then biennially, over 20 years. PDR was defined as grade $\geq$ 60 in one eye or < 60 but with panretinal photocoagulation scars consistent with laser therapy, according to the modified Airlie House system. Two haplotype blocks for the

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ADORA$_{2A}$ gene and two tagging single nucleotide polymorphisms (tSNPs) were selected using HAPMAP (rs2236624 and rs4822489)

Results: Univariate and multivariate logistic regression analysis of the rs2236624 tSNP revealed an association between the CT genotype and prevalent PDR at baseline, OR=1.68; (95%CI=1.11-2.54); p=0.03 and OR=2.17; (95%CI=1.24-3.81); p=0.03, respectively. Univariate and multivariate Cox proportional hazards regression analysis of rs2236624 revealed an association between the TT genotype and decreased incidence of PDR hazard ratio (HR) = .169; (95%CI=0.042); p=0.01 and HR = 0.101 (95%CI = 0.014-0.724), respectively. While the CT genotype was marginally associated with increased incidence of PDR in the univariate analysis HR=1.34; (95%CI:=0.96-1.87);p=0.09 and significantly associated with incidence of PDR in the multivariate analysis HR=1.54;(95%CI:=1.08-2.18);p=0.02.

Conclusions: Homozygosity for the TT genotype appears to be protective against PDR development, while heterozygosity increases PDR incidence.

5.2   ABSTRACT #2: VEGF

Purpose: The VEGF gene, particularly the 5 prime untranslated region has been implicated in diabetic retinopathy (DR), a microvascular complication of diabetes characterized by erratic vessel formation and visual impairment brought on by hypoxia and the damaging byproducts of glucose metabolism in individuals with diabetes. The purpose of this study was to investigate the VEGF gene within the context of proliferative diabetic retinopathy (PDR), the most severe form of DR, in a sample of patients with type 1 diabetes (T1D).

Methods: Participants of this study were drawn from the Pittsburgh Epidemiology of Diabetes Complications (EDC) prospective study of childhood onset T1D. The mean age was 28 years and the mean diabetes duration was 19 years for EDC participants at baseline. Participants had stereoscopic images of the retinal fundus at baseline (1986-1988) then biennially for 20 years. PDR was defined as grade $\geq$ 60 in one eye but < 60 but with panretinal photocoagulation scars consistent with laser therapy, according to the modified Arlie House system. Three haplotype blocks for the VEGF gene and 3 tagging single nucleotide polymorphisms (tSNPs) were selected using HAPMAP (rs2146326, rs833069 and rs10434). We decided to investigate two additional SNPs based on their significance in a similar population (rs3025021 and rs699947).

Results: Only rs10434 (GG) was marginally associated with PDR at baseline OR=1.29, (95% CI=0.75-2.26) and significantly associated with PDR in the multivariate analysis OR=2.47 (95% CI=1.15-5.29). Additional analysis revealed an association between rs10434 (AG) and hypertension [OR=2.13(95%CI=1.16-3.89); p=0.05]. These finding warrants further investigation as hypertension is a confounder, significantly associated with the rs10434 AG genotype and significantly associated with PDR, while, and the GG genotype of rs10434 is associated with PDR.

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6.0   RESEARCH PARTICIPANT RISK AND PROTECTION

6.1   HUMAN PARTICIPANTS

The Genetic Basis of Diabetic Retinopathy study consists of 496 (75.4%) of the 658 participants of the EDC study for whom genetic material was available.   The age of the participants at entry time of entry into the EDC study ranged from 8-48 years.   The mean age was 28 years.   Criteria for study participation required that all participants 1) were diagnosed with T1D prior to age 17 years, 2) lived 200 miles or within 2 and a half hour drive from Children's Hospital of Pittsburgh and 3) had no adult onset of any type of diabetes.

The specimens used in the study were collected by the Pittsburgh Epidemiology of Diabetes Complications (EDC) study.   The specimens were coded and de-identified.   The PI for the GBDR study had no connection to the EDC study data collection or the specimen collection sites.   She also had no access to any personal information or identifiers that could be used to link personal identifiers to the study participants.

6.2   VULNERABLE POPULATIONS

Inclusion of Children: This study is interested in the susceptibility to complications of diabetes, which typically does not occur until the subject has had the disease for some time.

Therefore the participants, diagnosed with diabetes as children, agreed to participate in the study however, informed consent was granted by their adult parents when the participant was recruited into the EDC study. Additionally all participants underwent a consent process for each EDC visit therefore as that became adults former childhood participants were able to grant their own informed consent for participation in the EDC study.

Inclusion of Women and Minorities: The data set for the GBDR study was an existing data set which consisted of the participants of the Pittsburgh Epidemiology of Diabetes Complications study (EDC); hence minority recruitment, age and gender of the study participants were pre-established. There were a total of 658 participants available for the GBDR study through the EDC study. The study population for the EDC consists of 325 (49.5%) female and 333 (50.5%) male participants, and the racial representation parallels that of the Allegheny County T1D registry being 4.4% African American and 95.4% Caucasian. None of the participants are of Hispanic, Asian, American Indian, Alaskan, Hawaiian or other descent. The percentage of African Americans in the EDC study population reflects the lower incidence of T1D in African-Americans as well as reflecting the population in the Pittsburgh area. Subjects enrolled in the EDC study were recruited from the Pittsburgh area within Allegheny County. The population found in this region is 84.3% white, 12.4% African American, 1.7% Asian, 0.3% some other race and 1.1% two or more races (Census, 2000). When reviewing these statistics, one should keep in mind that there is a higher incidence of type 1 diabetes in Caucasians than in other racial groups (ADA, 2003). Statistics communicating the number or percentage of African American's with diabetes are sparse; however 5% of reported cases of diabetes in African Americans are thought to be T1D (CDC, 2003). In the EDC there was no exclusion of potential participants based on race or gender, and the GBDR did not exclude participants based on race or

gender. All EDC participants for whom banked DNA were available were included in the GBDR study.

Source: The DNA samples used in this study were extracted from whole blood samples from participants of the EDC study.   The data base that was used was also from the PEDC study and contained de-identified information from study participants.

Recruitment: Participants for the EDC were recruited from the Allegheny County T1D Registry and the Children's Hospital of Pittsburgh Diabetes Registry. Retention efforts for the EDC included mail and telephone contacts. The GBDR study used banked DNA and an already existing data set and did not require resources for retention or recruitment.

Potential Risks: There were no risks to the subjects; however, there is the potential for great benefit from in that the results may potentially reveal a genetic predisposition to or protection from the development of proliferative diabetic retinopathy. Those involved with the study may also receive some professional benefit. The samples that were provided for the GBDR study were de-identified at another facility prior to being released for genotyping. All participants previously consented to having their genetic material investigated by researchers not initially directly involved with the EDC provided the samples were de-identified. All of the data generated from this study will be reported as aggregated data and the results of the study will not be revealed to the participants in the form of independent personalized genotyping results. All data generated from this study are housed in a database that is secured on a password protected computer.

7.0   SUMMARY OF STUDY

The purpose of this study was to investigate the genes involved with angiogenesis and vasculogenesis for a potential association with development of PDR. This project focuses on investigating the $ADORA_{2A}$ and the VEGF genes for this association. The investigation of each gene's association is described individually in detail in the two manuscripts contained within this document.

The study had two specific aims:

Specific Aim 1: Determine if a specific allele, specific genotype or haplotype generated for each candidate gene is associated with length of time to diagnosis PDR in individuals with T1D. This specific aim is addressed in the two manuscripts contained in this document, "The $ADORA_{2A}$ Gene is Associated with Proliferative Diabetic Retinopathy", and "Vascular Endothelial Growth Factor is Associated with Proliferative Diabetic Retinopathy and hypertension in Type 1 Diabetes". Survival analysis methods were used to investigate this specific aim in 2 year increments. There were no significant associations with regard to time to onset of PDR and the genotypes of the SNPs selected for the VEGF gene**;** however there were significant differences between genotypes of the ADORA gene and time to onset of PDR.

Specific Aim 2: Determine if a specific allele, specific genotype and/or haplotype generated for each candidate gene is associated with baseline prevalent of PDR. The planned analysis for this specific aim was altered from what had been described in the original proposal since most participants had some form of DR. This specific aim is addressed in the two manuscripts contained in this document, "The $ADORA_{2A}$ Gene is Associated with Proliferative Diabetic Retinopathy" and "Vascular Endothelial Growth Factor is Associated with Proliferative Diabetic Retinopathy and Hypertension in Type 1 Diabetes". Additionally to determine if there was a suppressor variable effect all genotypes that were not significantly associated with PDR were stratified into three groups, an "Early Onset" group identified as those who developed PDR after ≤25 years with T1D, a "Late Onset" group defined as those who did not develop PDR until after > 25 years with T1D, and a "Protected" group defined as those who did not develop PDR during their follow-up time in the EDC study.

Analysis of the rs10434 tSNP of the VEGF gene yielded an unanticipated finding. There was marginal association of the GG genotype with baseline prevalent PDR in the univariate analysis, but the significance of this association improved substantially in the multivariate analysis. Bivariate analysis of the covariates (included in the multivariate analysis) and the genotypes of the rs10434 tSNP found a significant association with baseline prevalent PDR but the associated confidence intervals did not inspire confidence in the results. This finding lead to post hoc, investigations of the covariates to determine if there was confounding, or an interaction between the variables. This directed the identification of an association between the GG genotype of the rs10434 tSNP of the VEGF gene PDR and the AG genotype of the same tSNP and hypertension.

Results:

ADORA$_{2A}$:

The CT genotype of rs2236624 tSNP of the ADORA$_{2A}$ gene was significantly associated with prevalence [OR=1.68; (95% CI: =1.11-2.54); p =0.03] and marginally associated with incidence [HR=1.34 (95%CI: =0.96-1.87); p=0.09] of PDR in the bivariate analysis. It was also significantly associated with both prevalence [OR=2.19; (95%CI: =1.24-3.86); p=0.038] and incidence [HR=1.54; (95%CI: =1.08-2.18); p=0.02] of PDR in the multivariate analysis after controlling for covariates. The TT genotype was associated with both protection from development of incident PDR in the bivariate [HR=0.17; (95%CI: =0.04-0.69); p=0.01] and in the multivariate [HR=0.01; (95% CI: =0.01-0.72); p=0.02] analysis after controlling for covariates. Analysis of the rs4822489 tSNP found an association between the GT genotype and PIPDR in the univariate analysis [HR=1.53; (95%CI: =1.12-2.09); p=0.008], and in the multivatiate analyses [HR=1.57; (95%CI: =1.13-2.17); p=0.0067] after controlling for covariates. To determine if there was increased risk associated with the 4822489 genotypes and PBPDR participants were stratified into late onset (PDR ≥25 years T1d duration), early onset (PDR<25 years T1D duration), and protected (No PDR during follow-up) groups. Those with the GT genotype were had a significantly increased association [OR=1.93; (95%CI: =1.11-3.35); p=0.03] with early onset PDR compared to the protected group. The association between the GT genotype of the rs4822489 tSNP was only marginally significant [OR=1.73; (95%CI: =0.89-3.36); p=0.12] in the multivatiate analyses after controlling for covariates.

The rs2236634 tSNP found to be associated with both baseline prevalent and incident PDR accounts for bases 23,166,024 through 23,168,324 of the ADORA$_{2A}$ gene. This region of

the gene codes for the portion of the g-coupled protein receptor that includes the 4th through the 7th transmembrane domains which includes several intracellular and extracellular domains as well as the entire c-terminus. Transmembranes 5-7 are highly conserved and are believed to play a fundamental role in ligand binding and recognition of agonist/antagonist complexes essential for exogenous manipulation of the receptor. The 22 amino acids bordering the 7th transmembrane are essential for protein folding and the cystine residue at position 394 and the argienine at position 309 are thought to allow the receptor to bind a multitude of accessory proteins not bound by other ARs. Furthermore this region also plays a part in mitogen activated protein kinase (MAPK) stimulation and receptor mobility and is influenced by cholesterol depletion in the cell membrane. Genomic variability in this portion of ADORA$_{2A}$ could result in variability in any of the above mentioned functions in this portion of the receptor. This variability appears to be explained by the C and T alleles tagging different haplotypes. Rs2236624 appears to be in linkage disequilibrium with 15 currently known SNPs including a non-synonymous SNP and a frame shift polymorphism.

The region tagged by rs4822489 spans bases 23,148,156-23,158,314. It includes the region of the receptor encompassing transmembranes 1-3. Transmembrane 3 encompasses aan amino acid region associated with ligand binding and may play a role in susceptibility to incident PDR, may be used as a target for pharmacogenetics, but requires further exploration.

Association of ADORA$_{2A}$ with PDR beyond the functions that this portion of the receptor performs may be nucleotide variability of the mRNA that could impact its stability and thereby receptor density. The effect of the tagged region of the gene on mRNA stability is currently unknown. Considering the fact, that adenosine is a byproduct of glucose metabolism one must

reason that variability in receptor function or availability may affect homeostatic mechanisms and biologic mechanisms involving adenosine particularly during periods of hyperglycemia.

Replicate studies for this gene need to be conducted. Provided the findings hold this information may lead us closer to the development of personalized medicine. Pharmacogenetics may be utilized to develope medical therapies for individuals at risk for PDR based on available information regarding this portion of the receptor and knowledge of ligands exerting agonist/antagonist effects. Nurses and other health care professionals could initiate earlier implementation of preventative therapy and more targeted patient teaching based on the patients genetic risk profile.

VEGF:

The rs10434 GG genotype was found to be marginally OR=1.23; (95%CI:-0.75-2.26) p=0.14 associated with the prevalence of PDR at baseline in the univariate analysis and significantly OR=2.40; (95%CI:=1.15-4.99); p=0.0065 associated with baseline prevalent PDR in the multivariate analysis. Interestingly, the rs10434 AG genotype was also associated with baseline hypertension as noted above. The other two tSNPS (rs833069 and rs2146323) and the SNP associated with PDR in the DCCT population (rs699947) were not associated with prevalent baseline PDR or baseline hypertension in the EDC population. None of the SNPs were associated with incident PDR. Homozygosity for the rs10434 tSNP is associated with baseline prevalent PDR in type 1 diabetes.

The rs10434 tSNP is the most 3 prime tSNP evaluated and it tags base 43,861,029-43,873,511 on the 5 prime to the 3 prime side of the gene respectively. This section of the gene starts in exon 8 and extends through the entire 3' untranslated region (3'UTR). Our findings do not implicate a specific VEGF isoform in susceptibility to PDR. The most plentiful isoforms of

the protein are the VEGF-121 amino acid isoform (excludes exons 6 and 7); the VEGF-165 amino acid isoform, (excludes exon 6); theVEGF-189 amino acid isoform, (includes all exons); and theVEGF-206 amino acid isoform, (includes all exons plus an additional 6'exon that is only found in this isoform). Our data supports association with the portion of the gene that houses exon 8 and given that all isoforms contain exon 8, our data does not support a specific isoform in susceptibility to PDR.

Exons 1-5 of the VEGF gene cover the receptor binding domain while exons 6-7 cover the heparin binding domain. The region implicated by our data excludes exons 1-7 of the gene. Investigators have found that portions of exons 7 and 8 working together promote VEGF binding to receptors that induce its biologic effects in the heparin binding domain; therefore the heparin binding domain cannot be excluded from implication in development of PDR since the region tagged by our tSNP excludes a physiologic tag for exon 7 does not exclude endogenous biochemical interactions that may require interaction between proteins produced by exon 7 and 8 acting together or synergistically that cannot be elucidated from physiologic marker tagging alone.

The 3'UTR of VEGF has 9 copies of the consensus AU rich element, which is associated with mRNA stability and is regulated by inadequately understood normal physiologic and pathologic processes. Proteins that increase mRNA stability in this region have been identified. Other endogenous proteins that have not been identified may bind to and increase mRNA stability of VEGF 3'UTR. Interestingly, binding sites associated with hypoxia have been identified and presence of hypoxia does increase VEGF expression. These characteristics of the region of VEGF tagged by the rs10434 tSNP provide a plausible explanation for the results of our findings in the GBDR population.

This study has implicated both the ADORA$_{2A}$ and the VEGF genes in PDR. These findings further support the heterogeneity involved with genetic susceptibility to PDR amongst individuals with T1D and offer increased understanding of the potential mechanisms involved with this susceptibility.

Strengths of this study included the fact that tSNPs were used to investigate the genes of interest. The selection of tSNPs ensured that segments representing the entire gene were considered for association with PDR. The investigation of this relatively homogenous population renders the results of this study generalizable to similar Caucasian populations with T1D in Western Pennsylvania. The prospective nature of this study and the wealth of data available in the database makes, the EDC an ideal population to investigate the complications of diabetes and the covariates associated with the development of these complications. Additionally the prospective nature of the longitudinal information contained in the database facilitates investigation of both cross-sectional baseline data and prospective data.

The strengths of the study are ironically the source of its limitations and areas for future investigation. Tagging SNPs take us to the region and possibly the actual location of the base change responsible for the phenotypes under investigation; however it does require that evaluation of the underlying variability in these regions be investigated before the actual variant responsible for the association is confirmed and since this next step is out of the scope of this current project, it is a limitation. This population was a rather homogenous population of Caucasian individuals with T1D; therefore, the results may not be generalized to other populations or to those with T2D.

APPENDIX A

ADORA$_{2A}$ ARTICLE

The ADORA$_{2A}$ Gene is Associated with Proliferative Diabetic Retinopathy

Bashira A. Charles, PhD(c), Trevor J. Orchard, M.D.,M. Med. Sci., Susan M. Sereika, PhD, Rachael G. Miller, MS, Janice S Dorman, PhD, Michael B. Gorin, PhD, Robert E. Ferrell, PhD, Yvette P. Conley, PhD

Abstract

Purpose: The ADORA$_{2A}$ receptor ameliorates many of the deleterious physiologic effects associated with tissue injury and biologic stressors, which may include those experienced by individuals with diabetes. We thus, investigated cross-sectionally and prospectively the association between the ADORA$_{2A}$ gene and proliferative diabetic retinopathy (PDR) in a sample of patients with type 1 diabetes (T1D).

Methods: Study participants were from the Pittsburgh Epidemiology of Diabetes Complications (EDC) prospective study of childhood onset (<17 years) T1D, (mean age 28 years, mean diabetes duration 19 years at enrollment). Participants underwent stereoscopic photography of the retinal fundus at baseline (1986-1988) then biennially, for 10 years and again at 18 years. PDR was defined as grade ≥ 60 in one eye or < 60 but with panretinal photocoagulation scars consistent with laser therapy, according to the modified Airlie House system. Two haplotype blocks for the $ADORA_{2A}$ gene and two tagging single nucleotide polymorphisms (tSNPs) were selected using HAPMAP (rs2236624 and rs4822489)

Results: Logistic regression analysis of the rs2236624 tSNP revealed an association between the CT genotype and prevalent PDR at baseline, both univariately [OR =1.67; 95%CI = (1.11 – 2.54); p=0.03] and multivariately following covariate adjustment [OR = 2.17; 95%CI = (1.24 – 3.81); p=0.03]. Univariate Cox proportional hazards regression (CPHR) analysis of rs2236624 found significant association between only the TT genotype and incident PDR [HR=0.17; 95%CI= (0.042-0.686); p=0.01]. Multivariate CPHR analyses found an association between the TT genotype [HR=0.10; 95%CI= (0.01-0.72); p=0.02] and the CT genotype [HR=1.54; 95%CI= (1.08-2.18); p=0.01] of rs2236624 and incident PDR. CPHR analysis of the rs4822489 tSNP found an association between the GT genotype and incident PDR in the univariate analysis [HR=1.53; 95%CI= (1.12-2.09); p=0.01], and in the multivariate analyses after controlling for covariates [HR=1.57; 95%CI= (1.13-2.17); p=0.01].

Conclusions: Homozygosity for the TT genotype of rs2236624 appears to be protective against PDR, while heterozygosity for rs2236624 and rs4822489 seems to increase PDR incidence.

The ADORA$_{2A}$ Gene is Associated with Proliferative Diabetic Retinopathy

<u>Introduction:</u>

Diabetic retinopathy (DR) is the cause of up to 24,000 incident cases of blindness in the United States each year, and is the most prevalent cause of blindness between the second and seventh decades of life (American Diabetes Association 2008). Most individuals with type 1 diabetes eventually experience some form of DR during the course of their lives; however, a greater number of patients with T1D develop proliferative diabetic retinopathy (PDR) than do patients with type 2 diabetes, 50% verses 10%, respectively (Klein, Klein et al. 1984; Frank 2004). Historically, it has been estimated that as many as 50% of individuals with T1D develop PDR after 15 years, and 60% develop PDR by 20 years (Klein, Klein et al. 1984) . A recent study of T1D reported a declining trend (from 38%-26.5%) in PDR development by 20 years of T1D duration, while 53% developed PDR after having had T1D for 25 years (Pambianco, Costacou et al. 2006). Studies have also shown that presence of DR and/or severity of DR attained tends to have characteristics consistent with heritability (Diabetes Control & Complications Trial/Epidemiology of Diabetes Interventions and Complications 2000; Rema, Saravanan et al. 2002; Hallman, Huber et al. 2005). The Family Investigation of Nephropathy and Diabetes Eye (FIND-Eye) study consisting of European Americans, African Americans and Mexican Americans assessed participants for susceptibility to

PDR using measures of heritability and found that polygenic heritability for PDR was roughly 25% (Arar, Freedman et al. 2008). Additionally there are racial differences in susceptibility to DR, in individuals from Latin, European and/or African descent (Diabetes Control and Complications Trial 1997; Harris, Klein et al. 1998; Tudor, Hamman et al. 1998; Trial 2000; Rema, Saravanan et al. 2002; Hallman, Huber et al. 2005; Arar, Freedman et al. 2008).

Adenosine is a powerful physiologic mediator thought to modulate cellular damage and the resulting tissue injury caused by biologic stressors. In the presence of oxidative stress (OS) hydrogen peroxide is formed in the cytosol rendering vascular endothelial cells more permeable (Hecquet, Ahmmed et al. 2008) and adenosine may ameliorate this process. Modulation by adenosine is directed by adenosine receptors (ARs), with adenosine A1 receptor having a proinflammatory response to tissue injury while the adenosine A2 receptor restricts inflammation and guards tissues from further damage (Salmon, Brogle et al. 1993; McKusick 2006). The rat model has provided evidence that ARs have variability in function and long term response to ischemic brain injury (Trincavelli, Melani et al. 2008). Other animal studies have provided evidence that $ADORA_{2A}$ protects the kidneys and the heart from ischemic reperfusion injury by reducing reactive oxygen species (ROS) generation, limiting mitochondrial damage and guarding against apoptosis (Yonehana and Gemba 1999; Xu, Mueller et al. 2005; Xu, Park et al. 2005). An experimental model using bovine retinal endothelial cells has provided some evidence that adenosine may play a role in up regulation of expression of a gene involved in glucose transport (GLUT1) (Takagi, Kinh et al. 1998).

It is postulated that reactive oxygen species (ROS) and oxidative stress (OS) caused by long term exposure to hyperglycemia may be responsible for some of the tissue damage associated with microvascular complications of diabetes. OS is also elevated in the retina of individuals with diabetes and may contribute to development of DR (Pan, Zhang et al. 2008). Factors other than OS that are ameliorated by $ADORA_{2A}$ and thought to contribute to development of DR are intraocular glycemia and speed of glucose metabolism out of the cell, hypoxia, and inflammation (Takagi, Kinh et al. 1998; McKusick 2006). Additionally, in humans, $ADORA_{2A}$ in vascular endothelial cells plays a part in vasodilation (Iwamoto, Umenmura et al. 1994).

The role $ADORA_{2A}$ plays in ameliorating effects of biologic stressors in humans and animals, coupled with the fact that adenosine's effects are modulated by ARs makes the $ADORA_{2A}$ gene a plausible candidate gene for exploring susceptibility to or protection from the development of PDR, although this has not been explored previously. $ADORA_{2A}$ may also affect severity of DR attained.   Thus, we investigated both cross-sectionally and prospectively the $ADORA_{2A}$ gene for association with PDR, the most severe form of DR, in a sample of patients with T1D.

Research Design and Methods:

Population:

Participants for this study were from the Pittsburgh Epidemiology of Diabetes Complications (EDC) prospective study of childhood onset (<17 years) T1D (baseline mean age 28 years and mean diabetes duration 19 years). All participants lived within 100 miles or a 2.5 hour drive from Pittsburgh at the time of recruitment and were members of the Children's Hospital of Pittsburgh diabetes registry which treated 70% of

all T1D patients in Allegheny County. Potential participants received a letter inviting them to have a physical examination and to complete several questionnaires. There were 979 eligible participants, 658 of whom (67.2%) participated in the entire EDC evaluation process (Kostraba, Klein et al. 1991; Costacou, Chang et al. 2006). The current study, "Genetic Basis of Diabetic Retinopathy" (GBDR), consists of 496 EDC participants for whom DNA was available (75.4%).

This study was approved by the Institutional Review Board at the University of Pittsburgh and written informed consent was obtained from participants prior to baseline data collection. The laboratory data collection methodologies for this population have been published previously (Orchard, Dorman et al. 1990; Lloyd, Klein et al. 1995; Costacou, Chang et al. 2006). Briefly, data collection relevant to the GBDR include baseline and biennial stereoscopic retinal exams, measurement of blood pressure (SBP, DBP), hypertension (HTN), total cholesterol (TCHOL), high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides (TRIG), glycosylated hemoglobin (GHb), body mass index (BMI), and documentation of ever smoker status (SMEVR) (Lloyd, Klein et al. 1995; Costacou, Chang et al. 2006).

Determination of Proliferative Diabetic Retinopathy (PDR):

Stereoscopic images of the retinal fundus were obtained for participants at baseline (1986-1988) then biennially, for those without PDR, over the course of 10 years and again at 18 years. Fields 1, 2 and 4 were taken using the Zeiss Camera and diagnosis and severity grading were based on assessment of these images by the Fundus Photography Reading Center at the University of Wisconsin using the Arlie House System. Three images were used instead of the standard 7 images, since it has

been previously validated that this gives acceptable results with good sensitivity and reliability. PDR was defined as either grade ≥ 60 in one eye or grade < 60 but with panretinal chorioretinal scars consistent with laser therapy, according to the modified Airlie House System. Baseline PDR was defined as the presence of PDR at the initial evaluation. Incident PDR was defined as PDR first diagnosed at a subsequent biennial follow-up time point.

Genotyping:

Two major haplotype blocks encompass the $ADORA_{2A}$ gene and two tagging single nucleotide polymorphisms (tSNPs) with a minor allele frequency of at least 20%, (rs2236624 and rs4822489), were selected to represent these blocks using HAPMAP (NCBI build 35) (HapMap Consortium 2003). The tSNPs were genotyped using TaqMan allele discrimination using the ABI Prism® 7000 Sequence Detection System with genotype assignment conducted with 2.0 ABI software (Applied Biosystems, Foster City, CA). The assay for each SNP was available through Applied Biosystems. The assay for rs2236624 was a made to order 40X assay while the assay for rs4822489 was an assay on demand 20X assay. The genotyping assays were conducted using the reagents and concentrations provided in the protocol supplied with the assays. The cycling conditions provided by Applied Biosystems are noted below; however, due to poor genotype cluster separation using this protocol, an extended protocol was used and generally resulted in improved separation and genotype discrimination.

TaqMan Conditions- Applied Biosystems Protocol:

- Step 1: 50ºC for 2 minutes UNG enzyme activation
- Step 2: 95ºC for 10 minutes AmpliTaq Gold
- Step 3: 95ºC for 15 seconds to denature DNA

- Step 4: 60ºC for 1 minute for annealing and strand extension

- Step 5: Repeat 30 times from Step 3

TaqMan Conditions- Extended Protocol:

- Step1: 50ºC for 2 minutes UNG enzyme activation

- Step 2: 95ºC for 10 minutes AmpliTaq Gold

- Step:3 95ºC for 15 seconds to denature DNA

- Step 4: 58ºC for 1 minute and 30 seconds for annealing and strand extension

- Step 5: Repeat 50 times from step 3

Statistical Analysis

The data were analyzed using SAS version 9.1.3 (SAS Institute, Inc., Cary,NC). The level of significance was established at .05 for two-sided hypothesis testing. Initially, Exploratory data analysis were performed to identify data abnormalities that may impact the validity of results. Univariate analysis of categorical predictor variables (hypertension, gender, smoking status, genotype) was conducted using contingency table analysis with the chi-square test for independence and univariate binary or polychotomous logistic regression. Exact methods were employed if cell sizes were small for these testing and modeling procedures. Continuous predictor variables (age, BMI, diabetes duration, GHb, HDL, LDL, TCHOL, TRIG) were analyzed using univariate binary or polychotomous logistic regression. Predictor variables found to be significant in the univariate analyses were included as covariates in subsequent multivariate analyses. . Those covariates found to be significant ($p<0.05$) or marginally significant ($0.05<p<.10$) in the multivariate analysis were included in the initial logistic regression model, while only those predictor variables found to be significant ($p=<0.05$) in the

multivariate analysis of the preliminary logistic regression model were used to construct the parsimonious model.

Kaplan-Meier product-limit estimation and the log-rank test were used to determine the crude relationship between genotypes and the time to the development of PDR in 5 year increments for diabetes duration and in 2.5 year increments using EDC follow-up time. Cox proportional hazards regression methods were used to prospectively examine the genotypes of the tSNPs for possible association with incident PDR over time. Cox proportional hazards regression model building strategies included forced entry methods to assess genotypes for association with incident PDR. Stepwise Cox proportional hazard regression (significance level of 0.05) methods were used to determine which predictor variables, including the tSNPs, were associated with incident PDR. Those predictor variables that had significant (p=<0.05) or had marginal levels of significance (0.05 < p < 0.10) were included in the preliminary predictive model. Stepwise Cox proportional hazards regression methods were used to prospectively analyze those significant and marginally significant predictor variables for association with incident PDR and to delineate the most parsimonious predictive model. For each model fitted residual analyses were conducted to identify sources of model misspecification and influential observations.

Results:

Demographic and Covariate Evaluations in EDC and GBDR Participants:

The EDC population at baseline consisted of 98% Caucasians and 51% male participants with 32% having prevalent PDR compared to 98% Caucasian, 51% male and 29% prevalent cases of PDR in the GBDR participants. Interestingly, there were significant differences between the subset of EDC participants without genetic material (n=172) and the GBDR participants on mean GHb, TCHOL, and LDL (Table 1).

GBDR Participants with Prevalent PDR Verses GBDR Participants with No PDR:

There were differences between GBDR participants with prevalent PDR and those who had not developed PDR at the time of their baseline evaluation (Table 2). Those with PDR tended to be older; have a longer duration of T1D; have a higher level of GHb; have a higher level of total cholesterol, LDL, and triglycerides; lower level of HDL; higher BMI; and greater prevalence of HTN (Table 2).

GBDR Participants with Incident PDR Verses No PDR:

There was no significant difference between GBDR participants and non-participants' mean diabetes duration, gender, HDL, or ever smoker status. However, GHb, HTN, TCHOL, LDL, TRIG, and BMI, differed significantly between GBDR participants with incident PDR and those who had not developed PDR during follow-up.

Table 1: Baseline Characteristics (mean and Standard Deviation (SD) or Number and Percent) GBDR Study Participants' Compared to EDC Non-Participants

Variable	EDC Participants with No Genetic Material (n=172) Mean (SD) or n (%)	EDC Participants in GBDR Study (n=486) Mean (SD) or n (%)	Degrees of Freedom	Test Statistic	p-value
Age (years)	28.1 (7.9)	27.4 (7.9)			
Duration (years)	20.1 (7.2)	19.1 (7.6)	1	F=2.23	.1354
Male	83 (48.3%)	250 (51.4%)	1	X^2=0.50	.4781
Glycosylated Hemoglobin (%)	10.8 (2.0)	10.3 (1.8)	1	F=13.59	.0002
Hypertension	33 (19.3%)	73 (15.2%)	1	X^2=1.07	.3013
Total Cholesterol (mg/dl)	200.9 (45.0)	188.2 (41.0)	1	F=10.56	.0012
High Density Lipoprotein (mg/dl)	53.1 (13.6)	54.1 (11.6)	1	F=0.70	.4015
Low density Lipoprotein	122.3 (38.1)	114.1 (33.6)	1	F=6.29	.0124
Triglycerides	133.2 (123.0)	101.3 (72.0)	1	F=17.48	<.0001*
Body Mass Index	23.4 (3.1)	23.6 (3.3)	1	F=0.83	.3621
Ever Smoker	75 (45.2%)	173 (36.4%)	1	X^2=3.24	.0719
Prevalent PDR	62 (36.0%)	142 (29.2%)	1	X^2=6.55	.0105
Incident PDR	46 (26.7%)	161 (33.1%)	1	X^2=1.72	.1900

*Significant interaction for group participation and age when age is ≥ 35.

Table 2: Baseline Characteristics (Mean and Standard Deviation (SD) or Number and Percent) of GBDR Study Participants by Prevalent PDR Status:

Variable	GBDR Prevalent PDR (n=142) Mean (±SD)	No PDR GBDR Study (n=183) Mean (±SD)	Degrees of Freedom	Test Statistic	p-value
Age (years)	37.2 (5.8)	24.8 (8.1)			
Duration (years)	25.1 (6.1) P=<0.0001	16.3 (7.1)	1	F=31.88	<0.0001
Male	79 (55.6%)	92 (50.3%)	1	X^2=0.56	0.4562
Glycosylated Hemoglobin	10.3 (1.8)	9.9 (1.7)	1	F=9.73	0.0020
Hypertension	51 (35.9%)	7 (3.8%)	1	X^2=25.62	<0.0001
Total Cholesterol (mg/dl)	204.9 (45.3)	173.3 (32.5)	1	F=27.49	0.0001
High Density Lipoprotein (mg/dl)	51.7 (11.3)	55.3 (12.0)	1	F=7.72	0.0058
Low Density Lipoprotein (mg/dl)	128.9 (38.0)	102.2 (26.6)	1	F=26.62	<0.0001
Triglycerides	120.8 (71.2)	81.1 (50.1)	1	F=19.57	<0.0001
Body Mass Index	24.2 (3.3)	22.9 (3.4)	1	F=7.97	0.0051
Ever Smoker	66 (47.5%)	61 (34.7%)	1	X^2=<0.01	0.9456

Table 3: Baseline characteristics for (mean ± Standard deviation or number and percent) of GBDR Study participants by Incident PDR Status:

Variable	GBDR Incident PDR (n=161) Mean (SD) or n (%)	No PDR GBDR Study (n=183) Mean (SD) or n (%)	Degrees of Freedom	Test Statistic	p-value
Age (yrs)	25.8 (6.9)	24.8 (8.1)			
Duration (yrs)	17.0 (6.3)	16.3 (7.1)	1	F=0.09	.7675
Male	79 (49.1%)	92 (50.3%)	1	X^2=0.0	.9132
Glycosylated Hemoglobin (%)	10.7 (1.9)	9.9 (1.7)	1	F=19.68	<.0001
Hypertension	15 (9.3%)	7 (3.8%)	1	X^2=4.08	.0435
Total Cholesterol (mg/dl)	190 (40.0)	173.3 (32.5)	1	F=17.45	<.0001
High Density Lipoprotein (mg/dl)	54.7 (12.1)	55.3 (12.0)	1	F=0.25	.6155
Low Density Lipoprotein (mg/dl)	114.3 (31.4)	102.2 (26.6)	1	F=13.03	.0004
Triglycerides	106.6 (87.0)	81.1 (50.1)	1	F=10.65	.0012
Body Mass Index	23.7 (3.0)	22.9 (3.4)	1	F=3.99	.0466
Ever Smoker	46 (28.8%)	61 (34.7%)	1	X^2=1.9721	.1602

Tagging SNP Analysis for Baseline PDR:

The allele proportions for the rs2236624 tSNP are 0.21 for T and 0.79 for C. The allele proportions for the rs4822489 tSNP are 0.61for G and 0.39 for T. Genotype counts and percentage with PDR at baseline are shown in Table 4 for both SNPs as well as results of contingency table analysis for prevalent PDR at baseline. The rs2236624 tSNP is in Hardy-Weinberg equilibrium (HWE) (X^2 = 1.47; df =1; p=.50);

while the 4822489 tSNP is not in HWE (X^2 = 5.61; df =1; p=.02).   There is some

evidence of heterozygote advantage possibly due to the disease enriched sample.

Table 4: Prevalent PDR Status by Genotype Distributions of tSNPs

	Rs2236624			Rs4822489		
Genotypes	CC	CT	TT	GG	GT	TT
GBDR Participants	308	154	24	195	212	84
%PDR	26	37	21	26	32	30
Total Undetermined	10 (<3%)			5 (<1%)		
Chi-Square df p-value*	X2=6.9076 df=2 p=.0316*			X2=1.7276 df=2 p=.4216		

*p-values reflect association of tSNP genotypes with prevalent PDR.

The distribution of prevalent PDR differed by genotype for the rs2236624 tSNP

but not the rs4822489 tSNP.   Using this modeling strategy diabetes duration,

hypertension, and LDL were identified as predictors of baseline prevalent PDR;

however, the CT genotype of rs2236624 was also significantly associated with prevalent

PDR at baseline.   This association was sustained after controlling for the covariates

listed in Tables 2 and 3.   The most parsimonious model is noted in Table 5.

Table 5: Stepwise Multivariate Logistic Regression Model for Baseline PDR (where the CC genotype is the tSNP reference)

Variable	Degrees of Freedom	Wald Chi-Square	p-value	Odds Ratio (OR)	95%CI for OR
Duration	1	61.56	<.0001	1.177	1.130-1.226
High Density Lipoprotein (mg/dl)	1	4.77	.0289	0.975	0.954-0.997
Hypertension (Y/N)	1	16.50	<.0001	3.900	2.022-7.520
Low Density Lipoprotein (mg/dl)	1	9.61	.0019	1.013	1.005-1.021
Ever Smoker (Y/N)	1	2.38	.1226	1.521	0.893-2.592
Rs2236624-TT vs.CC	1	0.79	.3732	0.824	0.226-2.998
Rs2236624-CT vs. CC	1	4.69	.0304	2.171	1.236-3.812

Tagging SNP Analysis for Incident PDR:

Genotypes of the rs2236624 tSNP were also associated with incident PDR (Table 6). Controlling for covariates associated with development of PDR from the univariate analysis and those indicated from the literature, genotypes of the rs2236624 tSNP remained significant. BMI and GHb were also significantly associated with the incidence of PDR.

Table 6: Stepwise Multivariate Cox Regression for Incident PDR tSNP (where the CC genotype is the tSNP reference)

Variable	Degrees of Freedom	Wald Chi Square	p-Value	Hazard Ratio (HR)	95%CI for HR
BMI	1	7.76	0.0053	1.071	1.020-1.124
GHB	1	18.57	<0.0001	1.213	1.111-1.324
NLDL	1	5.38	0.0204	1.006	1.001-1.011
Rs2236624-CT vs. CC	1	5.78	0.0162	1.536	1.082-2.181
Rs2236624-TT vs. CC	1	5.21	0.0225	0.101	0.014-0.724

Analysis of the rs4822489 tSNP found an association between the GT genotype and incident PDR in the univariate analysis [HR=1.53;(95%CI:=1.12-2.09);p=0.008], and this finding was sustained in the multivariate analyses [HR=1.57;(95%CI:=1.13-2.17);p=0.0067] after controlling for covariates (Table 7).

Table 7: Significant Multivariate Variables Associated with Incident PDR tSNP

Variable	Degrees of Freedom	Chi Square	p-Value	Hazard ratio (HR)	95%CI for HR
Rs4822489-GT vs. GG	1	7.35	.0067	1.568	1.133-2.171
Glycosylated Hemoglobin	1	16.21	<.0001	1.193	1.095-1.300
Low Density Lipoprotein	1	7.59	.0059	1.007	1.002-1.012

Tagging SNP Analysis for Early Onset PDR, Late Onset PDR and Protected from PDR Groupings

We expanded our analyses of the rs4822489 tSNP in light of our findings to determine if it is associated with PDR risk. GBDR participants were stratified into three groups; Early Onset (PDR <25 years T1D duration); Late Onset (PDR ≥ 25 years T1D duration); and Protected (no PDR ≥ 25 years T1D duration). Exploring the association between those with early onset versus no PDR during follow-up revealed an association between the rs4822489 genotype and prevalence of PDR; however, this relationship was diminished in the multivariate analysis (Table 8).

Table 8: Rs4822489 (n=254) Early Onset PDR vs. Protection from PDR Development

Binary Logistic Regression with the GG Genotype as the Reference Group					
Variable	Degrees of Freedom	Wald Chi Square	p-value	Odds Ratio (OR)	95%CI for OR
4822489-TT	1	0.40	.5249	1.116	0.543-2.293
4822489-GT	1	4.72	.0299	1.927	1.107-3.354
Multivariate Logistic Regression with the GG Genotype Used as the Reference Group					
GHB	1	14.78	.0001	1.458	1.203-1.766
HTN	1	10.88	.0010	12.915	2.824-59.061
LDL	1	8.92	.0028	1.018	1.006-1.031
4822489-TT	1	0.71	.3981	0.926	0.394-2.176
4822489-GT	1	3.01	.0830	1.716	0.886-3.324

Discussion

Our findings suggest that the region of the $ADORA_{2A}$ gene tagged by the rs2236624 SNP is associated with susceptibility to PDR in the GBDR study participants. he CT genotype is associated with increased risk of PDR at baseline as well as incident PDR, while the TT genotype is associated with decreased risk of incident PDR.

The ADORA$_{2A}$ gene spans from base 23,159,092-23,168,324 at chromosome location 22q11.23. It is part of the G-protein coupled receptor super family and is expressed in the basil ganglia, blood vessels, platelets and other tissues in the body.   It encompasses 19 introns and 22 exons at the genomic level while the mature protein product is coded for by 1 intron and 2 exons (Hubbard, Aken et al. 2007; Maglott, Ostell et al. 2007).

The rs2236624 tSNP accounts for bases 23,166,024 through 23,168,324 of the ADORA2a gene. This region of the gene begins in an intron and extends through the second of the two exons involved in the final protein product. This region of the gene codes for the portion of the receptor that includes the 4th through the 7th transmembrane domains which includes several intracellular and extracellular domains as well as the entire C terminus. (Vastrik, D'Eustachio et al. 2007).

The region of the ADORA$_{2A}$ that appears to be involved in PDR has been characterized and the functions it performs may provide plausible mechanisms for the associations found in this study. Transmembrane domains 5-7 are highly conserved and are thought to play a vital role in ligand binding and recognition of agonist/antagonist complexes crucial for exogenous manipulation of the receptor (Olah and Stiles 2000).   Using neuroblastoma cells investigators found that the ADORA$_{2A}$ and D2-dopamine receptors located in the cell membrane sometimes act antagonistically while at other times they work synergistically enhancing the positive effects of adenosine. ADORA$_{2A}$ has a 122 amino acid intracellulear terminal C tail that is 3 times the length of other GCPRs with the sector bordering the 7th transmembrane domain (22amino acids) being essential to appropriate protein folding while the remaining 100

amino acids are not essential for G-protein coupling or ligand binding. ADORA$_{2A}$ houses a cystine residue at position 394 and an argienine at position 309 which are thought to allow the receptor to bind with a host of accessory proteins not bound by the other ARs (Zezula and Freissmuth 2008). Additionally, this region of the receptor is involved in mitogen activated protein kinase (MAPK) stimulation and receptor mobility that is influenced by cholesterol depletion in the cell membrane (Charalambous, Gsandtner et al. 2007).

Variability at the genomic level for this portion of ADORA$_{2A}$ could result in variability in any of the above mentioned functions of this portion of the receptor. This variability appears to be explained by the C and T alleles of rs2236624 tagging different haplotypes. Rs2236624 appears to be in linkage disequilibrium with 15 currently known SNPs and any underlying variability tagged by rs2236624 could alter receptor function. Four of these SNPs are within exons but have synonymous coding, 3 are intronic, 6 lie in the 3'untranslated region, one is a non-synonymous SNP and another is a frame shift polymorphism. The association of ADORA$_{2A}$ with PDR beyond the functions that this portion of the receptor performs may be due to nucleotide variability of the mRNA impacting its stability, and thereby receptor density. The effect of the tagged region of the gene on mRNA stability is currently unknown. Fully evaluating this segment of the gene for underlying variability as well as functional characterization of this variability on receptor function or mRNA stability are potential areas of further investigation.

The region tagged by rs4822489, implicated in incident PDR, spans bases 23,148,156-23,158,314. It includes the region of the receptor encompassing transmembranes 1-3. Transmembrane 3 encompasses an amino acid region

associated with ligand binding that may play a role in susceptibility to incident PDR. This region may be used as a target for pharmacologic intervention, following further exploration (Olah and Stiles 2000).

ADORA$_{2A}$ stimulation has been linked to psychomotor depression, sleep induction, immune-suppression, and vasodilation, while its suppression has been linked to hypertension, aggression, and inflammation, alleviation of symptoms of ETOH withdrawal, and amelioration of neurotoxicity (Yu, Frith et al. 2004). There is evidence that ADORA$_{2A}$ activation decreases the expression of vascular endothelial growth factor, a primary mitogen associated with development DR (Olah and Roudabush 2000). Experimental models have also shown that the ADORA$_{2A}$ receptor plays a role in glucose transport, vasodilation, prevention of apoptosis, hypoxia, inflammation and resolution of inflammation (Takagi, Kinh et al. 1998; Lee and Emala 2001; Xu, Mueller et al. 2005; Frobert, Hanink et al. 2006; McKusick 2006).

This study evaluated the ADORA$_{2A}$ gene however, there are four members of the AR family and the potential role of these other three genes in PDR is another area for further investigation. The ADORA$_1$ plays a significant role in the pro-inflammatory responses of the cell while ADORA$_{2A}$, and ADORA$_{2B}$, play a significant role in limiting the cells response to inflammation, thereby producing an anti-inflammatory response. While the ADORA$_3$ plays a role in the cell's response to ischemia (McKusick 2006).

This study was limited by the fact that the population was largely homogenous consisting of 100% T1D patients who were 98% Caucasian. The fact that the mean diabetes duration was 19 years at time of entry into the study limited our ability to explore the natural history of DR in reference to the genotype with which one is

endowed, since 50% of individuals with T1D develop PDR after 15 years and 60% develop PDR after 20 years. While the participants in our study did have a lower incidence of PDR with approximately 27% of the EDC population developing PDR after 20 years with T1D and 53% developing PDR after 25 years with T1D, this effect is likely due to improved glycemic control. Additionally 85% of the EDC participants had some form of DR at the time of entry into the study. This gave us the opportunity to explore the effect of genetic endowment on advancement to development of PDR. It would be beneficial to further explore the $ADORA_{2A}$ gene in another in a population of individuals with T2D as well as in another racial/ethnic group.

This study implicates the $ADORA_{2A}$ gene in PDR in individuals with childhood onset T1D. This association is limited to the portion of the gene characterized above. The functions performed by this receptor, its impact on factors known to be important to susceptibility to DR, and the implications of the AR family on susceptibility to diabetes provide feasible mechanisms for this association. While replication of these finding is paramount to moving forward with this line of investigation, these findings spark interest in all of the ARs as playing a role in diabetes complications and suggests that $ADORA_{2A}$ may be a therapeutic target to improve protection from complications among individuals with diabetes.

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APPENDIX B

VEGF ARTICLE

Vascular Endothelial Growth Factor is Associated with Proliferative Diabetic

Retinopathy and Hypertension in Type 1 Diabetes

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Abstract

Purpose: The VEGF gene, particularly the 5 prime untranslated region has been implicated in diabetic retinopathy (DR), a microvascular complication of diabetes characterized by erratic vessel formation and visual impairment brought on by hypoxia and the damaging byproducts of glucose metabolism in individuals with diabetes.   The purpose of this study was to investigate the VEGF gene within the context of proliferative diabetic retinopathy (PDR), the most severe form of DR, in a sample of patients with type 1 diabetes (T1D).

Methods: Participants of this study were drawn from the Pittsburgh Epidemiology of Diabetes Complications (EDC) prospective study of childhood onset T1D (mean age 28 years and mean diabetes duration 19 years at baseline).   Participants had stereoscopic images of the retinal fundus at baseline (1986-1988) then biennially for 20 years.   PDR was defined as grade $\geq$ 60 in one eye but < 60 but with panretinal photocoagulation scars consistent with laser therapy, according to the modified Arlie House System. Three haplotype blocks for the VEGF gene and corresponding tagging single nucleotide polymorphisms (tSNPs) were selected using HAPMAP (NCBI Build 36: rs2146326, rs833069 and rs10434).   We decided to investigate two additional SNPs based on their significance in a similar population (rs3025021 and rs699947).

Results: Univariately, rs10434 (GG) was marginally associated with PDR at baseline [OR=1.299; 95% CI= (0.747-2.260); p=0.136], but significantly associated with prevalent PDR in the multivariate analyses [OR=2.401 95% CI= (1.153-4.99); p=0.007].   Due to evidence of possible interaction or confounding; additional analyses were conducted and revealed an association between rs10434 (AG genotype) and hypertension at baseline

No significant associations were observed for rs10434 with incident PDR. No significant associations were found for rs2146323, rs833069, rs3025021, and rs699947 with prevalent or incident PDR.

Conclusion: The region of the VEGF gene tagged by the rs10434 SNP is associated with susceptibility to PDR and hypertension in this population, a region of the gene not previously associated with DR.

Introduction

Vascular endothelial growth factor (VEGF) has been implicated in the pathogenesis of diabetic retinopathy (DR), a microvascular complication of diabetes characterized by erratic vessel formation and visual impairment brought on by hypoxia and the damaging byproducts of glucose metabolism in individuals with diabetes (Sheetz and King 2002; Penn, Madan et al. 2008). VEGF is a necessary component of normal angiogenesis. It plays a role in wound healing and pathologic processes such as retinopathies. VEGF engineers angiogenesis via initiation of vascular endothelial cell proliferation, migration, and tube formation (Favard, Moukadiri et al. 1991; McKusick 2006; Penn, Madan et al. 2008). Retinal hypoxia is a component of the pathological response to the hyperglycemic state and VEGF is up-regulated in the presence of hypoxia (Campochiaro 2000; Perrin, Konopatskaya et al. 2005). Oxidative stress and inflammation are part of the pathologic response to hyperglycemia and these processes also result in the up-regulation of VEGF (Penn, Madan et al. 2008), which is a primary trigger for the neoangiogenesis and the weak leaky vessels characterized by DR

(Perrin, Konopatskaya et al. 2005). Furthermore, VEGF has been well defined in both animal and human models as contributing to the development of DR.

Starting in the early 1990s animal and human studies identified an increase in VEGF level in the retina and the vitreous fluid of rats, primates and humans with DR (Aiello, Avery et al. 1994; Miller, Adamis et al. 1994; Nakagawa, Chen et al. 2000). Later, genetic association studies reported significant associations between VEGF variants in the promoter region of the gene and prevalence of some form of DR or PDR across populations with T1D and T2D (Suganthalakshmi, Anand et al. 2006; Uhlmann, Kovacs et al. 2006; Nakamura, Iwasaki et al. 2008).   In 2002 investigators reported an association between PDR and the 5'-untranslated region, specifically the C-634 allele of the VEGF gene, in a population of 268 Japanese patients with T2D [OR=7.7; 95%CI= (1.8-30.9); p=.002] (Awata, Inoue et al. 2002). Subsequently, investigators in England reported an association between the VEGF-460C allele and development of PDR in a Caucasian population of individuals with T1D and T2D [OR 2.5; 95%CI= (1.20-5.23); p0.027] (Ray, Mishra et al. 2004). In a population of 210 T2D patients of east Indian ancestry investigators found that the C-7T, the T-1498C, and the C-634G, polymorphisms of the VEGF gene were significantly associated with DR [OR=4.37; 95%CI= (2.44-7.84); p0.0001] (Suganthalakshmi, Anand et al. 2006).   Interestingly, Nakamura and colleagues found a significant association between SNP-2578C/A of the VEGF gene and PDR with the AA genotype at SNP-2578 being strongly associated with PDR and with DR after controlling for diabetes duration.   They found no association for the C-634G polymorphism of the VEGF gene and prevalence of DR   (Nakamura, Iwasaki et al. 2008).   Investigators of the Diabetes Control and Complications Trial

(DCCT) provided evidence that three SNPs (rs3025007, rs3025020 and rs3025035) of the VEGF gene were associated with severe retinopathy in a univariate model, while eight SNPs (rs699947, rs833070, rs2146323, rs3035007, rs3025010, rs3025020, rs3025021, and rs3025028) were associated with severe retinopathy in the multivariate analyses, (Al-Kateb, Mirea et al. 2007) potentially indicating a suppressor effect for SNPs not identified in the univariate analyses. Finally, there is evidence that two SNPs in the promoter region of VEGF, rs13207351, specifically the CC and the AA genotype, and rs1570360, specifically the AA genotype, were significantly associated with PDR in a mixed population of T1D and T2D patients (Churchill, Carter et al. 2008).

We decided to investigate the entire VEGF gene and its flanking regions using tSNPs and did so in a longitudinal cohort where incident PDR could be evaluated.

Research Design and Methodology:

Population:

This study consists of participants of the Pittsburgh Epidemiology of Diabetes Complications (EDC) prospective study of childhood onset (<17 years of age) T1D. Mean age and diabetes duration at time of entry into the study were 28 years and 19 years, respectively.   Inclusion criteria for the study required that participants 1) were members of the Children's Hospital of Pittsburgh Diabetes Registry or Allegheny County Type 1 Diabetes Registry and 2) live within 100 miles or within a two and a half hour drive from Pittsburgh.   Nine hundred seventy-nine eligible registrants were sent a letter inviting them to have a physical exam and to fill out several questionnaires. Six hundred fifty-eight (67.2%) took part in the full EDC evaluation process.   Participants of the

current study, "The Genetic Basis of Diabetic Retinopathy" (GBDR), are made up of 496 (75.4%) individuals for whom genetic material was available. A more thorough description of the methods have been previously published (Kostraba, Klein et al. 1991; Costacou, Chang et al. 2006).

The Institutional Review Board at the University of Pittsburgh approved this study and written informed consent was acquired from participants preceding baseline data collection. Procedures for laboratory data collection for participants of this study have been described in prior publications (Orchard, Dorman et al. 1990; Lloyd, Klein et al. 1995; Costacou, Chang et al. 2006). In short, data collection consists of baseline and biennial stereoscopic retinal fundus exams, measurement of blood pressure (SBP, DBP), documentation of hypertension (HTN) defined as SBP ≥ 140 and/or DBP ≥ 90, total cholesterol (TCHOL), high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides (TRIG), glycosylated hemoglobin (HGB), body mass index (BMI), and documentation of smoking status (Lloyd, Klein et al. 1995; Costacou, Chang et al. 2006). Potential covariates were selected based on variables reported in the literature to be associated with PDR and included variables found to be significant based on findings of the univariate analysis.

Genotyping:

Three tSNPs with a minor allele frequency of at least 20% and representing the three main haplotype blocks of VEGF were selected via HAPMAP (NCBI build 36; rs833069, rs2146323 and rs10434). After reviewing the findings of the DCCT, which consisted primarily of a population of Caucasian individuals with T1D very similar to the demographics of our cohort, we decided to explore two additional SNPs (rs699947 and

rs3025021) associated with PDR in the DCCT; however the rs3025021 assay failed to provide genotype data and was excluded from analyses. Removal of this SNP did not impact our evaluation of the entire VEGF gene given that we were already investigating tSNPs and all of those assays worked well.

The tSNPs were genotyped using TaqMan allele discrimination using the ABI Prism® 7000 Sequence Detection System with genotype assignment conducted with 2.0 ABI software (Applied Biosystems, Foster City, CA). The assay for each SNP was available through Applied Biosystems (All assays were made to order 40X assays). The genotyping assays were conducted using the reagents and concentrations provided in the protocol supplied with the assays. The cycling conditions provided by Applied Biosystems are noted below and were used for rs699947, rs2146323 and rs8333069; however due to poor genotype cluster separation using this protocol, an extended protocol was used for rs10434 and resulted in improved separation and genotype discrimination.

TaqMan Conditions- Applied Biosystems Protocol:

- Step 1: 50ºC for 2 minutes UNG enzyme activation

- Step 2: 95ºC for 10 minutes AmpliTaq Gold

- Step 3: 95ºC for 15 seconds to denature DNA

- Step 4: 60ºC for 1 minute for annealing and strand extension

- Step 5: Repeat 30 times from Step 3

TaqMan Conditions- Extended Protocol:

- Step1: 50ºC for 2 minutes UNG enzyme activation

- Step 2: 95ºC for 10 minutes AmpliTaq Gold

- Step:3 95ºC for 15 seconds to denature DNA

- Step 4: 58ºC for 1 minute and 30 seconds for annealing and strand extension

- Step 5: Repeat 50 times from step 3

PDR Defined:

Stereoscopic images of the retinal fundus were obtained for participants at baseline (1986-1988) then biennially, for those without PDR, over the course of 10 years and again at 18 years. Fields 1, 2 and 4 were taken using the Zeiss Camera and diagnosis and severity grading were based on assessment of these images by the Fundus Photography Reading Center, at the University of Wisconsin using the Arlie House System. Three images were used instead of the standard 7 images, since it has been previously validated that this gives acceptable results with good sensitivity and reliability. PDR was defined as either grade ≥ 60 in one eye or grade < 60 but with panretinal chorioretinal scars consistent with laser therapy, according to the modified Airlie House System. Baseline PDR is defined as the presence of PDR at the initial evaluation. Incident PDR was defined as PDR diagnosed at a subsequent biennial follow-up time point.

Analyses:

Hardy-Weinberg equilibrium was evaluated for each SNP separately. Data were analyzed using SAS version 9.1.3 (SAS Institute, Ins., Cary, NC). P-values less than 0.05 were considered statistically significant.   Analysis of univariate categorical variables (hypertension, gender, smoking status and genotype) were conducted using logistic regression, chi-square goodness of fit or exact chi square if cell sizes were small.   Continuous variables age, body mass index (BMI), diabetes duration,

glycosylated hemoglobin (GHb), high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides (TRIG) were analyzed using univariate, binary or polychotomous logistic regression. Multivariate analyses were conducted using binary and multivariate logistic regression. Variables that achieved levels of significance or near significance (<0.20) in the univariate analysis were included in the multivariate analysis as covariates. Variables previously reported in the literature as being associated with PDR were also included as covariates in the analysis.

Association between the SNPs and prevalence of PDR at baseline were determined using bivariate logistic regression. Association between the SNPs and prevalence of PDR at baseline controlling for covariates was determined using multivariate logistic regression methods. Cox proportional hazards regression methods were used to prospectively assess incident PDR in relation to the participants' SNP type. Model building strategies consisted of forced entry and or backward regression strategies using covariates found to be significant or near significant in the univariate analysis. Predictor variables found to be significant or near significant in the univariate analysis were included in the preliminary model. Predictor variables found to be significant or marginally significant in the preliminary model were included in the final model or the most parsimonious model. Backward regression models were used to analyze SNPs bivariately for association with PDR. Forward multivariate Cox proportional hazards regression methods were used to prospectively analyze tSNPs for an association with PDR controlling for covariates.

Post Hoc Analyses

Given the increased association between the GG genotype of rs10434 in the multivariate analyses we decided to conduct additional analyses using logistic regression to further explore these findings. Additionally chi square goodness of fit test was used to assess categorical predictor variables for association with rs10434; while, general linear modeling was used to assess the continuous dependent variables for association with rs10434 genotypes.

Results:

Demographic and Covariate Evaluations in EDC and GBDR Participants:

The EDC population consists of 98% Caucasians, is 49% female, with 32% having prevalent PDR, while the GBDR participants consists of 98% Caucasians, and 49% female, with 29% having prevalent PDR. Interestingly, there were significant differences between the subset of EDC participants without genetic material (n=172) and the GBDR participants on mean GHb, TCHOL, and LDL (Table 1). Within the GBDR population there were differences in baseline characteristics between those with prevalent PDR and those who had not developed PDR at the time of baseline evaluation (see Tables 2 and 3). Those with PDR tended to be older; have a longer duration of T1D; have a higher level of GHb; have higher levels of total cholesterol, LDL, and triglycerides; lower level of HDL; higher BMI; and greater prevalence of HTN (Table 2 and 3).

Table 1: Baseline Characteristics (Mean and Standard Deviation (SD) or Number and Percent) GBDR Study Participants' Compared to EDC Non-Participants

Variable	EDC Participants with No Genetic Material (n=172) Mean (SD or n (%)	EDC Participants in GBDR Study (n=486) Mean (SD or n (%)	Degrees of Freedom	F-value	Wald Chi Square	p-value
Age (years)	28.1 (7.9)	27.4 (7.9)				
Duration (years)	20.1 (7.2)	19.1 (7.6)	1	2.23		.1354
Male	83 (48.3%)	250 (51.4%)	1	NA	0.50	.4781
Glycosylated Hemoglobin (%)	10.8 (2.0)	10.3 (1.8)	1	13.59	NA	.0002
Hypertension	33 (19.3%)	73 (15.2%)	1	NA	1.07	.3013
Total Cholesterol (mg/dl)	200.9 (45.0)	188.2 (41.0)	1	10.56	NA	.0012
High Density Lipoprotein (mg/dl)	53.1 (13.6)	54.1 (11.6)	1	0.70	NA	.4015
Low density Lipoprotein	122.3 (38.1)	114.1 (33.6)	1	6.29	NA	.0124
Triglycerides	133.2 (123.0)	101.3 (72.0)	1	17.48	NA	<.0001*
Body Mass Index	23.4 (3.1)	23.6 (3.3)	1	0.83	NA	.3621
Ever Smoker	75 (45.2%)	173 (36.4%)	1	NA	3.24	.0719
Prevalent PDR	62 (36.04%)	142 (29.22%)	1	NA	6.55	.0105
PDR	46 (26.74%)	161 (33.13%)	1	NA	1.72	.1900

*Significant interaction for group participation and age when age is ≥ 35.
p-values reflect between group comparisons

Table 2: Baseline Characteristics (Mean and Standard Deviation (SD) or Number and Percent) of GBDR Study Participants by Prevalent PDR Status

Variable	GBDR Prevalent PDR (n=142) Mean (SD)	No PDR GBDR Study (n=183) Mean (SD)	Degrees of Freedom	F-Value	Wald Chi-Square	p-value
Age (years)	37.2 (5.8)	24.8 (8.1)	1			
Duration (years)	25.1 (6.1)	16.3 (7.1)	1	31.88	NA	<.0001
Male	79 (55.6%)	92 (50.3%)	1	NA	0.56	.4562
Glycosylated Hemoglobin (%)	10.3 (1.8)	9.9 (1.7)	1	9.73	NA	.0020
Hypertension	51 (35.9%)	7 (3.8%)	1	NA	25.62	<.0001
Total Cholesterol (mg/dl)	204.9 (45.3)	173.3 (32.5)	1	27.49	NA	.0001
High Density Lipoprotein (mg/dl)	51.7 (11.3)	55.3 (12.0)	1	7.72	NA	.0058
Low Density Lipoprotein (mg/dl)	128.9 (38.0)	102.2 (26.6)	1	26.62	NA	<.0001
Triglycerides	120.8 (71.2)	81.1 (50.1)	1	19.57	NA	<.0001
Body Mass Index	24.2 (3.3)	22.9 (3.4)	1	7.97	NA	.0051
Ever Smoker	66 (47.5%)	61 (34.7%)	1	NA	<.01	.9456

Table 3: Baseline characteristics for (mean ± Standard deviation or Number and Percent) of GBDR Study participants by Incident PDR Status

Variable	GBDR Incident PDR (n=161) Mean (±SD)	No PDR GBDR Study (n=183) Mean (±SD)	Degrees of Freedom	F-Value	Wald Chi-Square	p-value
Age (years)	25.8 (6.9)	24.8 (8.1)				
Duration (years)	17.0 (6.3)	16.3 (7.1)	1	0.09	NA	.7675
Male	79 (49.1%)	92 (50.3%)	1	NA	0.01	.9132
Glycosylated Hemoglobin	10.7 (1.9)	9.9 (1.7)	1	19.68	NA	<.0001
Hypertension	15 (9.3%)	7 (3.8%)	1	4.08	NA	.0435
Total Cholesterol (mg/dl)	190 (40.0)	173.3 (32.5)	1	17.45	NA	<.0001
High Density Lipoprotein (mg/dl)	54.7 (12.1)	55.3 (12.0)	1	0.25	NA	.6155
Low Density Lipoprotein (mg/dl)	114.3 (31.4)	102.2 (26.6)	1	13.03	NA	.0004
Triglycerides	106.6 (87.0)	81.1 (50.1)	1	10.65	NA	.0012
Body Mass Index	23.7 (3.0)	22.9 (3.4)	1	3.99	NA	.0466
Ever Smoker	46 (28.8%)	61 (34.7)	1	1.97	NA	.1602

SNP Analysis:

All four of the VEGF polymorphisms investigated were in HWE. Only rs10434 resulted in significant findings. No associations were found between prevalent PDR and rs833069 (p=.4732), rs699947 (p=.8576), and rs2146323 (p=.6701) (see Table 9). Additionally, no association was found between incident PDR and rs833069 (p=.8540), rs699947 (p=.8801) and rs2146323 (p=.5904) (see Table 9). This lack of association was maintained after controlling for covariates. All subsequent analyses were conducted only with rs10434.

Binary logistic analysis revealed a marginal association between rs10434 and PDR [OR=1.3, 95% CI = (0.75-2.3) and OR=0.87 95%CI= (0.52-1.5)] for the GG and the AG genotypes, respectively. Controlling for covariates using the full covariate model revealed a significant association between diabetes duration (p = <.0001), HDL (p = .04), HTN (<.0001), LDL (p=.005) and the homozygous (GG) genotype ((p=.0005). The most parsimonious model which included the above noted variables revealed significant associations between prevalent PDR and duration, HDL, LDL, HTN, rs10434 (GG) genotype (see Table 4).

Table 4: Variables Associated with Baseline Prevalent PDR

Variable	Degrees of Freedom	Wald Chi-Square	p-value	Odds Ratio (OR)	95%CI for OR
rs10434-GG vs. AA	1	7.40	.0065	2.401	1.154-4.999
Rs10434-AG vs. AA	1	1.10	.2932	1.172	0.595-2.309
DUR	1	66.88	<.0001	1.177	1.132-1.224
HTN	1	15.29	<.0001	3.675	1.914-7.075
HDL	1	6.88	.0087	0.971	0.949-0.993
LDL	1	11.04	.0009	1.013	1.005-1.021

Bivariate Cox regression analysis of the genotypes of rs10434 for association with PDR found that there was no association between the AG genotype [HR=0.928; 95%CI= (0.625-1.379); p=0.7110)] and the GG genotype [HR=0.907; 95%CI= (0.560-1.418); p=.6683], using the AA genotype as the reference.

Post Hoc Analysis of rs10434:

The association between PDR and the GG genotype of rs10434 controlling independently for duration, HTN, LDL, and HDL was evaluated. In the analysis of duration and LDL the p-value for the GG genotype remained significant; however the 95% CI was suboptimal; while in the analysis of HTN and LDL, the genotype achieved marginal significance (see Tables 5-8). We therefore decided to explore the covariates for an association with the rs10434 genotype.

Table 5: Analysis of rs10434 Genotype Controlling for Diabetes Duration with Prevalent PDR at baseline:

Variable	Degrees of Freedom	Wald Chi-Square Value	p-value	Odds Ratio (OR)	95% CI for OR
Rs10434-GG	1	4.58	.0323	1.735	0.909-3.313
Rs10434-AG	1	1.50	.2203	0.983	0.539-1.791
Diabetes Duration	1	97.43	<.0001	1.193	1.152-1.236

Table 6: Analysis of rs10434 Genotype Controlling for Hypertension with Prevalent PDR at Baseline

Variable	Degrees of Freedom	Wald chi-Square Value	p-value	Odds Ratio (OR)	95% CI for OR
Rs10434-GG	1	3.46	.0627	1.672	0.907-3.085
Rs10434-AG	1	0.32	.5729	1.142	0.643-2.026
Hypertension	1	54.63	<.0001	8.161	4.677-14.241

Table 7: Analysis of rs10434 Genotype Controlling for LDL with PDR

Variable	Degrees of Freedom	Wald Chi Square	p-value	Odds Ratio (OR)	95% CI for OR
Rs10434-GG	1	3.90	.0484	1.545	0.850-2.807
Rs10434-AG	1	1.73	.1883	0.929	0.530-1.628
LDL	1	31.74	<.0001	1.020	1.013-1.027

Table 8: Analysis of rs10434 Genotype Controlling for HDL with PDR

Variable	Degrees of Freedom	Wald Chi-Square	p-value	Odds Ratio (OR)	95%CI for OR
Rs10434-GG	1	2.76	0.0968	1.366	0.777-2.402
Rs10434-AG	1	1.82	0.1769	0.883	0.520-1.498
HDL	1	9.86	0.0017	0.971	0.954-0.989

Logistic regression was used to assess the relationship between the rs10434 tSNP and the categorical variable hypertension. There was no significant association between the rs10434 genotype and diabetes duration, LDL, or HDL. Interestingly, this analysis revealed a significant association between rs10434 and hypertension with the AG genotype showing a direct association with hypertension while the GG genotype did not (see Table 9).

Table 9: Summary VEGF Genotype Frequencies and Associations with Baseline PDR, Hypertension and Incident PDR

SNP/Genotype	Frequency	Baseline PDR Count (%) ChiSq	Incident PDR Count (%) ChiSq	Hypertension Count (%) ChiSq
Rs10434				
AA	102	29 (28)	36 (35)	23 (23)
AG	241	62 (26)	82 (34)	29 (12)
GG	141	48 (34)	43 (31)	21 (15)
Chi Square		.22	.89	.05
Rs833069				
CC	66	15 (23)	23 (35)	8 (12)
CT	212	64 (30)	71 (33)	31 (15)
TT	212	64 (30)	66 (31)	33 (16)
Chi-Square p-value		.47	.85	.81
Rs699947				
AA	101	31 (31)	31 (31)	16 (16)
AC	233	65 (28)	78 (33)	36 (15)
CC	148	44 (30)	50 (34)	19 (13)
Chi-Square p-value		.86	.88	.73
Rs2146323				
AA	52	17 (33)	14 (27)	7 (14)
AC	210	62 (30)	67 (32)	34 (16)
CC	227	64 (39)	80 (35)	32 (14)
Chi-Square p-value		.81	.59	.81

Discussion:

The outcomes of our study suggest that the region of the VEGF gene tagged by the rs10434 SNP is associated with susceptibility to PDR and hypertension in the GBDR study participants. The GG genotype is marginally associated with increased PDR at baseline in the univariate analysis but significantly associated with PDR in the multivariate analysis, while the AG genotype is significantly associated with hypertension at baseline in the univariate analysis. The rs10434 tSNP is the most 3 prime tSNP evaluated and LD appears to deteriorate around nucleotide 43,861,029 on the 5 prime side and around 43,873,511 on the 3 prime side (HapMap 2006). This section of the gene originates in exon 8 and encompasses the 3 prime untranslated region (3'UTR) (Onesto, Berra et al. 2004; HapMap 2006).

Our results do not implicate a specific VEGF isoform in susceptibility to PDR. The most plentiful isoforms of the protein are the VEGF-121 amino acid isoform (excludes exons 6 and 7); the VEGF-165 amino acid isoform, (excludes exon 6); theVEGF-189 amino acid isoform, (includes all exons); and theVEGF-206 amino acid isoform, (includes all exons plus an additional 6'exon that is only found in this isoform). Our data supports association with the portion of the gene that houses exon 8 and given that all isoforms contain exon 8, our data does not support a specific isoform in susceptibility to PDR. Exon 8 has been associated with vascular morphogenesis (Ruhrberg, Gerhardt et al. 2002) and attenuation of spontaneous hypertension in rats (Vilar, Waeckel et al. 2008). This is interesting given our data that supports an independent association of rs10434 with hypertension.

VEGF receptor binding is not likely to be involved in susceptibility to PDR but heparin binding may play a role. Exons 1-5 of the gene cover the receptor binding domain while exons 6-7 cover the heparin binding domain (Penn, Madan et al. 2008), both domains are not associated with PDR in our population. The region implicated by our data excludes exons 1-7 of the gene, however heparin binding cannot be ruled out as a mechanism for our association given that investigators have found that portions of exons 7 and 8 working together promote VEGF binding to receptors that induce its biologic effects in the heparin binding domain (Jia, Bagherzadeh et al. 2006; von Wronski, Raju et al. 2006).

VEGF transcript stability may play a role in susceptibility to PDR. The 3'UTR is primarily responsible for VEGF mRNA stability and regulation and the entire 3'UTR is implicated by our data. This region has 9 copies of the consensus AU rich element, which is associated with mRNA stability and is controlled by inadequately understood normal physiologic and pathologic processes. Three proteins that increase stability of mRNA in this region, have been identified; protein-interacting protein 2, HuR of the ELAV family, and hnRNPL, a ribonucleoprotein. Other endogenous proteins may bind to and increase the stability of VEGF 3'UTR mRNA; however they have not yet been identified. Interestingly, binding sites associated with hypoxia have been identified (Onesto, Berra et al. 2004) and the presence of hypoxia does increase VEGF expression (Penn, Madan et al. 2008). These characteristics of the region of the VEGF gene tagged by the rs10434 SNP provide a plausible explanation for the results of our findings in the GBDR population.

Several previous studies have implicated the 5'region of VEGF with PDR; however our data does not support a role for the 5 prime region of the gene. Churchill and colleagues conducted a case-control study of 45 individuals with type 1 or 2 diabetes and 61 controls with T1D or T2D but no PDR for genetic association with VEGF. These investigators analyzed SNPs located in the 5'untranslated region and intronic SNPs encompassing the VEGF gene. They found the rs2146323 tSNP to be associated with PDR (Churchill, Carter et al. 2008). Possible reasons for the differences in our studies may be the respective population sizes. Additionally their population consisted of both T1D and T2D patients who tend to have different rates of PDR development. The diabetes duration of the cases ranged from 7-44 years while controls diabetes duration ranged from 14-50 years and covariates such as hypertension were not considered. Interestingly our results are similar to those of Finnish investigators who found no association between rs2146223 and PDR (Liinamaa 2007).

In a study of 349 Caucasians with T2D, investigators found that while VEGF levels were elevated in the vitreous fluid and blood of individuals with PDR there was no genetic association with rs2010963 (located in the 5'untranslated region of the VEGF gene) and PDR. They controlled for hypertension (cross-sectional) but did not find an association between PDR and hypertension (Petrovic, Korosec et al. 2008). Investigators of the Diabetes Control and Complications trial (DCCT) did not find an association between the rs699947 SNP and PDR in their univariate analysis. Interestingly they did find an association between the SNP and PDR in their multivariate analysis. These findings suggest that there may be a yet unidentified covariate effect

related to the rs699947 SNP's genotypes and PDR, similar to the relationship we found between the rs10434 tSNP, hypertension and PDR. Awata et al investigated 268 Japanese patients with T2D for an association between DR and the 5'untranslated region of the VEGF gene. They did find an association between non proliferative DR and the 5'untranslated region of VEGF, however they did not control for hypertension or diabetes duration (Awata, Inoue et al. 2002). Their findings were supported by a study of Caucasians patients with T1D and T2D; however this study was limited by the fact that there were no controls without DR (Ray, Mishra et al. 2004).

The elevated levels of VEGF, erratic vessel formation and genetic association studies linking VEGF to DR has lead to the use of VEGF suppressors, such as Pegaptanib and Ranibizumab, as a therapeutic intervention for the treatment of retinopathies. There has been some success associated with these therapies; however given VEGF's heterogeneity and pleiotropic effects the suppression of VEGF is also associated with hypertension, impaired wound healing, proteinuria disrupted capillary vessel formation, disrupted bone growth, infertility and impaired muscle restoration and cardio-adaptive processes (Simo and Hernandez 2008). Low dose statins such as Simvastatin have been found to increase reparative VEGF production leading to normal oxygen levels in the retina and inhibition of pathologic vessel growth. Interestingly, higher doses resulted in pathologic neovascularization, retinal ischemia and endothelial cell apoptosis (Reinhold, O'Neill et al. 2008). While there are side effects to these drugs, the success that has been noted in treatment of retinopathy also supports that

level of VEGF, controlled via transcript stability, is a viable hypothesis to support the association of the 3'UTR of the VEGF gene in susceptibility to PDR.

This study has provided additional information regarding VEGF and the development of PDR. Unlike other studies it has implicated the 3'UTR of the VEGF gene. This study is strengthened by the fact that it used tSNPs that are representative of the entire VEGF gene to investigate the association of VEGF with PDR. While the DCCT investigators did investigate the 5'UTR and intronic SNPs spanning the entire VEGF gene they did not use tSNPs and may not have fully covered the 3'UTR tagged by rs10434.

All of these studies are building our knowledge of the role of VEGF in the development of DR and is adding to our knowledge of its pleiotropic effects and may help in the development of more specific gene based therapeutic interventions. These studies are also moving us closer to being able to provide patients with customized preventative care and tailored therapeutic interventions through a better understanding of the mechanisms involved with susceptibility to DR, particularly PDR.

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